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— SECTION D —

# ZOOLOGICAL SCIENCES

## *Contents*


Page

A Cytochemical Study of the Absorption and Distribution of Iron in the Frog, *Rana pipiens*—*David J. McCallion and Joseph L. Scott* - - - - - - - - - - - - - - 119

The Thyroid Gland in Relation to the Seaward Migration of Pacific Salmon—*William S. Hoar and G. Mary Bell* - - - 126

Deposition of $P^{32}$ in Developing *Drosophila*—*R. L. B. Irwin, J. W. T. Spinks, and T. J. Arnason* - - - - - - - 137

The Fate of Phenothiazine in the Gastrointestinal Tract of the Sheep—*R. P. Harpur, W. E. Swales, and O. F. Denstedt* - - 143

Absorption of Phenothiazine from the Rumen of Sheep, and its Estimation in Blood Serum—*R. P. Harpur, O. F. Denstedt, and W. E. Swales* - - - - - - - - - - - - - - 162

The Morphology and Development of the Sheep Nematode, *Strongyloides papillosus* (Wedl, 1856)—*M. A. Basir* - - 173

The Ticks of Alberta with Special Reference to Distribution—*John H. Brown and Glen M. Kohls* - - - - - - - 197



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# Canadian Journal of Research

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VOL. 28, SEC. D. JUNE, 1950 NUMBER 3


## A CYTOCHEMICAL STUDY OF THE ABSORPTION AND DISTRIBUTION OF IRON IN THE FROG, *RANA PIPIENS*[1]


BY DAVID J. MCCALLION[2] AND JOSEPH L. SCOTT[3]



### Abstract

Cytochemical studies for iron in starved frogs fed a single large dose (50 to 200 mgm.) of ferric sulphate or metallic iron showed that free iron was most readily absorbed by the intestinal mucosa of the duodenum. Liver iron increased slightly two hours after feeding, reached a maximum condition after six hours


CORRECTION

On p. 59 of the April issue of this section (Section D, Volume 28, Number 2) please insert the following in place of the first paragraph under the heading **The Mosquito Species at Churchill.**

The following species of mosquitoes have now been recorded as occurring in the vicinity of Churchill, Man.:

Culicinae:

*Aedes nearcticus* Dyar
*nigripes* Zett.
*punctor* Kby.
*communis* DeG.
*excrucians* Wlk.
*campestris* D. & K.
*Aedes flavescens* Müller
*cinereus* Meig.
**spencerii* Theo.
**riparius* D. & K.
*Culiseta alaskaensis* Ludl.
*impatiens* Wlk.

Chaoborinae:

*Chaoborus nyblaei* Zett.
**crystallinus* DeG.
**Eucorethra underwoodi* Und.
**Mochlonyx culiciformis* DeG.

### Materials and Experimental Methods

During the early part of April, large adult *Rana pipiens* were used within a few days after their arrival from North Carolina. We have assumed that the frogs were in a starved condition since they had been recently taken from hibernation and their fat bodies were depleted.


[1] *Manuscript received December 28, 1949.*
*Contribution from the Department of Biology, Brown University, Providence, R.I.*



[2] *Teaching Fellow, Brown University (Now Assistant Professor, Acadia University, Wolfville, N.S.).*

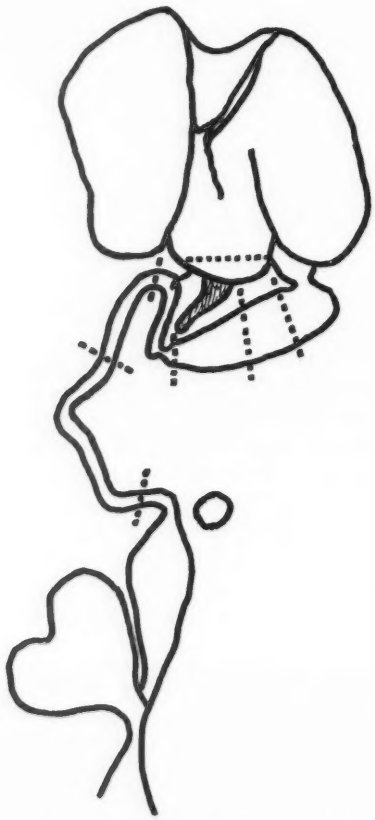
[3] *Teaching Fellow, Brown University.*



[The April issue of Section D (Can. J. Research, D, 28:45-117. 1950) was issued April 20, 1950.]

The work was divided into two parts. The first experiment included four control and 12 treated animals. Six of the experimental animals were each fed approximately 200 mgm. of very finely powdered metallic iron in a small gelatin capsule. The capsule was placed as far back in the pharynx as possible and the mouth was held closed until the capsule was swallowed. One hour was allowed for the capsule to dissolve, then two frogs were killed each time after 2, 6, and 12 hr.

Similarly, each of six additional frogs was fed 200 mgm. of ferric sulphate mixed with pieces of ground beef. Two frogs were killed each time after 2, 6, and 12 hr.



TEXT-FIG. 1. *A diagram of the ventral aspect of the digestive system of the frog. Broken lines indicate the levels at which sections were taken for cytochemical study.*

All of the animals were killed by pithing. Three circumferential portions of the stomach, and of the small intestine, and one piece of the pars posterior of the left lobe of the liver were taken for the study of iron (Text-fig. 1).

In the second experiment we used two normal controls and four experimental animals. To each of two experimental frogs, after MS222 (tricaine methanesulphonate) anaesthesia, was administered one ml. of 5% solution of ferric sulphate (50 mgm.) directly into the stomach with a glass pipette. An equal amount of the ferric sulphate solution was injected into the body cavity of each of the two other frogs. All the animals were killed eight hours after treatment. In this experiment iron was studied in the spleen and kidney as well as in the liver and portions of the digestive tract.

## Techniques

All of the tissues were fixed in Bouin's fluid for 12 to 24 hr. They were dehydrated through dioxane, embedded in paraffin, and sectioned at 5$\mu$. The deparaffinized sections were coated with collodion to prevent the loss of sections. Whenever possible, control and treated slides were processed together.

For the demonstration of ionic iron two techniques were applied to companion sections of each tissue; Gomori's (6) variant of the Berlin blue (Prussian blue) method of Perls, and Humphrey's (10) dinitrosoresorcinol. Sections treated with Gomori's method were usually counterstained with carmalum.

To demonstrate bound (masked) iron, sections of the liver, kidney, and spleen were treated with Macallum's method (from Bensley and Bensley (1) ). The iron is released by exposure to 4% sulphuric acid in 95% alcohol at 60° C. for four hours. This is followed with the Prussian blue test (equal parts of 1% hydrochloric acid and 1% potassium ferrocyanide for three minutes).

## Observations

### A. *The Distribution of Iron in Starved Control Frogs*

No free iron was found in the mucosa of the gastrointestinal tract in three of the four control animals. However, in the fourth control there was a slight trace of free iron in the duodenal mucosa. The lumen of the gut, posterior to the opening of the bile duct, contained a considerable amount of bile. Varying, but small, amounts of free iron were detected in the liver, always localized only in the stellate cells around the pigment masses (Fig. 1). In the spleen also, iron was associated with the patches of pigment (Fig. 3), but there were, in addition, traces in the macrophages. There was no evidence of free iron in the kidney.

The Macallum technique revealed bound iron in the nuclei of all the cells of the liver and spleen. There was none in the nuclei of the kidney cells.

### B. *The Distribution of Iron after Injection or Feeding of Ferric Sulphate Solution*

Eight hours after introducing 50 mgm. of the iron sulphate in solution into the stomach, free iron was localized only in the mucosa of the duodenum.

The columnar epithelial cells were filled with iron diffusely distributed throughout the cell (Fig. 5). Clusters of deeply stained granules were localized supranuclearly, in the region usually occupied by the Golgi apparatus (Fig. 6). The rest of the gastrointestinal tract showed no iron. There was a pronounced increase in the iron content in the liver and spleen but not in the kidney. In the liver, free iron was found in the Kuppfer cells, in the lining cells of the sinusoids, and in cells associated with the patches of pigment (Figs. 2, 7). In the spleen, increased amounts of free iron were found in the pigmented areas and in the macrophages (Fig. 4). Masked iron was found in the nuclei of the cells of the liver, spleen, and kidney. In the liver the reaction was stronger in the nuclei of the Kuppfer cells and of the lining cells of the sinusoids. In the spleen it was strong in the nuclei of all cells. In the kidney, the nuclei of the cells of the glomeruli and uriniferous tubules gave good reactions. Although all three of these structures showed appreciable increases in bound iron over the controls, the increase was most striking in the kidney (Fig. 8), where in the control animals our tests were negative.

After intraperitoneal injection of ferric sulphate, the distribution of both free and bound iron is not significantly different from that outlined above. However, the quantities of free iron in both the liver and spleen are smaller. Very large amounts of iron were found in the subserosa histiocytes of the organs studied. In some cases, free iron had penetrated into the organ. There was some iron in the muscularis of the gut but none in the mucosa.

## Explanation of Figures

*For the demonstration of ionic iron, Gomori's variant of the Berlin (Prussian blue) method of Perls was applied to the materials shown in Figs. 1 to 7 inclusive. For the material shown in Fig. 8, Macallum's technique was used to reveal bound iron.*

Fig. 1. *Liver of control frog. The large patches of pigment usually contain small amounts of free iron. Only the pigment cells contain small amounts of demonstrable free iron. 390×*

Fig. 2. *Liver of frog eight hours after receiving 50 mgm. of ferric sulphate in solution. Kuppfer cells show dense deposits of free iron. Iron deposits in pigment patches (P) are also increased. 390×*

Fig. 3. *Spleen of control frog showing pigment patches. Free iron is usually limited to pigment patches. 390×*

Fig. 4. *Spleen of frog eight hours after receiving 50 mgm. of ferric sulphate in solution. Note the dense deposits of free iron found in the macrophages. The iron deposits in pigment patches (P) are also markedly increased. 390×*

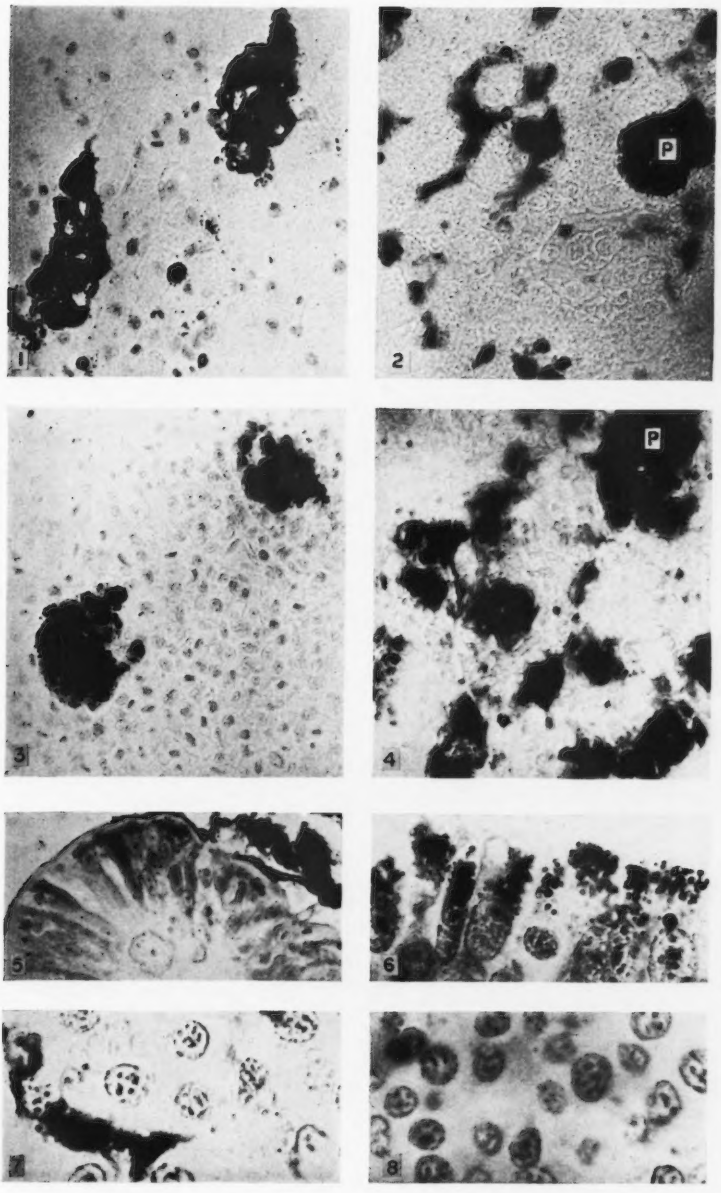
Fig. 5. *Duodenal mucosa of frog eight hours after receiving 50 mgm. of ferric sulphate in solution. Note large concentration of free iron in portion of cells next to the lumen. 390×*

Fig. 6. *A higher magnification of the duodenal mucosa counterstained with carmalum. Free iron is conspicuously localized in the supranuclear region of these cells. 870×*

Fig. 7. *Free iron in the Kuppfer cell of the liver of a frog eight hours after the feeding of 50 mgm. of ferric sulphate in solution. The preparation was counterstained with carmalum. 870×*

Fig. 8. *Kidney cells of a frog eight hours after an intraperitoneal injection of 50 mgm. of ferric sulphate. Note the distribution of bound iron in the nuclei. 870×*

PLATE I



C. *The Distribution of Iron in Frogs after the Feeding of Ferric Sulphate or Reduced Iron*

These observations substantiate the results of the experiment just described. The region of the digestive tract most active in iron absorption appeared to be the duodenum. The iron salt was absorbed more quickly than the reduced iron. Two hours after the ingestion of ferric sulphate the heaviest concentration of iron appeared in the epithelium of the duodenum; after 6 and 12 hr. there was a marked decrease in demonstrable iron. After ingestion of metallic iron, on the other hand, increasing amounts of iron appeared in this same region in animals killed after 2, 6, and 12 hr. The greatest amounts of demonstrable iron appeared 12 hr. after the ingestion. With the higher dosages used (200 mgm.) in this experiment, there were suggestions that some iron might be absorbed through the mucosa of the stomach. Traces of free iron were found in the mucosa cells of the lower region of the stomach after the administration of ferric sulphate. After the ingestion of metallic iron there was an increase of demonstrable iron over the entire stomach mucosa.

As in the preceding experiment, the amount of iron in the liver was greatly increased. Here the results obtained, after either form of iron was used, were more alike. Maximum concentrations were obtained after six hours. After 12 hr., the amount of iron present in the liver was not as great as at six hours. Although after 6 or 12 hr. the amount of demonstrable iron in the livers of frogs that received either iron salts or reduced iron appeared to be the same, the increase in iron appeared earlier in animals that received iron salts. While at the end of two hours the animals receiving iron salts showed a definite increase in liver iron, those receiving reduced iron contained only a moderate increase over the normal untreated animals.

The results of these two experiments indicate that following the administration of iron, increased amounts of ionic iron were first detected in the cells associated with the pigment patches, and next in the Kuppfer and lining cells of the sinusoids of the liver.

## Discussion

Our observations on the normal distribution of iron in the tissues of *Rana pipiens* in general correspond with the reports of earlier workers on related forms. In control frogs, free iron is associated with pigment deposits of liver and spleen. It is present in some macrophages of the spleen and only rarely in the Kuppfer cells of the liver. Negative results were obtained for the other parts of the liver and spleen, and for all the regions of the gastrointestinal tract and the kidney. In untreated *Rana temporaria*, during the summer months, Makarov (11) found iron in the Kuppfer cells but not in the parenchyma of the liver or in the cells of the kidney, pancreas, or intestine. Berg (2) described a positive Turnbull reaction in the newly formed pigment granules of pigment cells and in the macrophages of the liver and spleen of the same species, but not in the parenchymatous cells of the liver.

De Robertis (5) associated ionic iron with the newly formed yellow pigment and bound iron (by microincineration) with the older brown pigment in the liver of *Bufo arenarum*. He also observed that when pigment was scarce it was limited to the Kuppfer cells, while, when more abundant, it occupied the parenchymatous cells. Chlopin (3) demonstrated that in the livers of frogs killed during the winter months iron is found only rarely in the Kuppfer cells and never in the parenchymatous cells.

Makarov (11) and Chlopin (3) greatly increased the iron content in frog tissues by injecting solutions of iron salts into the dorsal lymph sac. The Kuppfer cells and the pigment cells of the liver became laden with iron after a single injection of 0.5 cc. of 5% solution of iron saccharide (Chlopin). When injections were administered daily for four days, iron deposits were extended to the liver parenchyma cells (Makarov (11) ). After this treatment, iron appeared in the cells of the pancreas, in the mucosa of the intestine, and in the glomeruli and uriniferous tubules. Wherever found, iron was associated with the Golgi apparatus of the cells. In our experiments, we did not detect free iron in the parenchyma cells of the liver, in the cells of the kidney, or in the mucosa of the intestine. The differences in dosage, length of treatment, and method of administration of the iron salts may be responsible for this discrepancy. There was, however, an increase in bound iron in the cells of the liver, spleen, and kidney.

Concerning the role of the duodenum in iron absorption, the studies of Welch, Wakefield, and Adams (14) suggest that absorption of iron in man is selective, thus preventing an excessive accumulation of iron. Absorption is increased appreciably in anemia. Hahn (9) envisions the existence of an acceptor mechanism in the gastrointestinal mucosa, which, when saturated under normal conditions, allows only minimal absorption from the lumen of the gut. Michaelis, Coryell, and Granick (12) have shown this mechanism to be ferritin. They find that the quantity of ferritin in the gastrointestinal tract is greatest in the duodenum. This may account for the greater absorption of iron in this region. During iron deficiencies ferritin is increased in the duodenal mucosa. In spite of the dramatic increase in free iron in the tissues of animals treated by us, it is evident that only a minimal amount of the iron introduced into the frogs was absorbed by the gastrointestinal mucosa. In view of the massive amounts of iron used in these experiments, perhaps these data demonstrate the presence of the acceptor mechanism of Hahn (9).

## Acknowledgment

We are indebted to Dr. William Montagna, under whose direction this work was carried out, for his continued enthusiastic interest and many helpful suggestions.

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# THE THYROID GLAND IN RELATION TO THE SEAWARD MIGRATION OF PACIFIC SALMON[1]

By William S. Hoar[2] and G. Mary Bell[3]

## Abstract

Histological examination of the thyroid glands from chum salmon fry taken in the river, estuary, or sea shows the organ to be in a quiescent condition at the time of migration. If, however, this species is retained in fresh water for two or three months the gland becomes extremely hyperplastic. The pink salmon thyroid behaves in essentially the same way as that of the chum, but migrating pink fry taken at great distances from the sea have active glands. The thyroids of yearling coho and sockeye moving into the sea display heightened activity. Thyroid activity is apparently greater in coho migrants taken later in the season from the headwaters of rivers. In part, the heightened thyroid activity seen in these migrating Pacific salmon is probably a spring-time seasonal change. It seems, however, to be more particularly related to the increased metabolic work of osmotic regulation and salt balance in a fish physiologically prepared for life in the sea. In general, this study suggests that the increased thyroid activity seen in young migrating salmonoids is largely due to increased demands for thyroid hormone in the metabolism of a fish no longer completely adjusted physiologically to fresh water.

## Introduction

Heightened thyroid activity has been demonstrated in seaward migrants of Atlantic salmon (11, 5) and in rainbow trout smolt moving into lakes (21). It would be interesting to know whether the thyroid hormone controls the smolt transformation and migration or whether the observed changes are due to quite different factors. There is experimental evidence to suggest that the thyroid is responsible for at least some of the changes associated with the development of the smolt stage (15, 22). However, the season (11), diet (18), or salinity of the environment (20) may alter the thyroid picture in teleosts, and changes observed in the gland, at the time of migration, might be due to factors such as these and might not be related directly to the migration.

The Pacific salmon (genus *Oncorhynchus*) with their variable life histories and times of migration offer interesting material for a comparative approach to the problem of thyroid activity and salmon migration. Chum salmon (*Oncorhynchus keta*) and pink salmon (*O. gorbuscha*) always migrate to sea soon after emerging from the gravel. Sockeye (*O. nerka*), on the other hand, very rarely move into the sea as fry but usually migrate into a lake at this stage to spend one year (sometimes longer) there before going to the sea. Coho (*O. kisutch*) live one year—rarely two— in the streams before undertaking their migration to salt water. The spring salmon (*O. tschawytscha*) is more variable than the other species spending from a few months to one year in the

[1] *Manuscript received January 31, 1950.*

*Contribution from the Zoology Department, University of British Columbia, Vancouver, B.C. with financial assistance from the National Research Council of Canada.*

[2] *Professor of Zoology and Fisheries.*

[3] *Research Assistant.*

fresh water. In respect to time of seaward migration the latter two species most closely resemble the Atlantic salmon and steelhead (rainbow) trout.

In this paper the histophysiology of the thyroid gland of the different species of Pacific salmon migrants is compared and an attempt made to evaluate the role of the thyroid in relation to the movement from fresh to salt water.

## Materials and Methods

Most of the material was collected at Fort Babine, B.C. by W. R. Hourston and at Port John, B.C. by G. J. Hunter. Some additional material was procured at Lake Cowichan, B.C. by V. S. Black and at Nile Creek by Ferris Neave. The writers are indebted to them and to R. E. Foerster, Director, Pacific Biological Station for cooperation in obtaining this material and providing certain facilities at the Station.

The tissues were fixed in Bouin's formol-picric-acetic acid fixative and prepared for histological study by routine methods. Tissues were sectioned serially and haematoxylin and eosin were used as stains throughout. Table I summarizes the material examined histologically. Throughout this paper measurements are for total length.

TABLE I

NUMBER OF THYROIDS EXAMINED HISTOLOGICALLY

| Length of fish, mm. | Number of thyroids | | | | |
|---|---|---|---|---|---|
| | Chum | Pink | Sockeye | Coho | Spring |
| 30 – 40 | 7 | 9 | 3 | 3 | 2 |
| 40 – 80 | 7 | 5 | | 7 | |
| 80 – 160 | | | 3 | 6 | |

Methods used in the experiments are given in connection with their description.

## Results

*Pacific Salmon Fry (30 mm. to 40 mm.)*

Pink and chum salmon always migrate to salt water during this stage of development and sockeye usually move into lakes. If the thyroid controls the migratory behavior of these salmonoids, distinct differences in microscopic anatomy might be expected to appear in this stage. This is not the case.

*Chum Fry*

Four of the seven specimens studied were from fresh water, two from the estuary, and one from the sea.

The histological picture in all specimens is that of a quiescent or very mildly active gland. The follicles are relatively small in size and spherical in shape. The epithelium is cuboidal to low cuboidal and the colloidal material strongly acidophilic and refractive (Figs. 1 and 2). The mass of tissue is relatively greater than in some species (Fig. 6 and Table II) but there is no evidence of the heightened activity described for *Salmo salar* at the time of migration (11).

TABLE II

CROSS SECTIONAL AREAS $\left(\frac{MM^2}{150}\right)$ OF THYROIDS THROUGH LEVELS INDICATED IN THE RECONSTRUCTIONS OF FIGS. 6, 7, AND 8

| Species | Length, mm. | Habitat | Level | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| Chum | 41 | Fresh water | 149 | 221 | 48 |
| Chum* | 42 | Sea | 117 | 180 | 106 |
| Pink | 38 | Fresh water | 143 | 195 | 215 |
| Sockeye | 31 | Fresh water | 15 | 17 | 35 |
| Coho | 39 | Fresh water | 41 | 105 | 60 |
| Spring | 40 | Fresh water | 39 | 501 | 379 |
| Chum | 65 | Fresh water | 1503 | 2323 | 910 |
| Chum* | 67 | Sea | 929 | 1000 | 369 |
| Coho | 63 | Fresh water | 257 | 1241 | 240 |
| Coho* | 74 | Migrant | 619 | 1338 | 697 |
| Pink* | 68 | Sea | 432 | 622 | 126 |

* *Not figured.*

*Pink Fry*

Six of the thyroids in this group were from fish taken in fresh water and three from fish in the sea. The histological picture is somewhat variable. In all three fish from the sea the gland is quiescent. In two of the freshwater fish (from Fort Babine) the gland shows evidence of activity in the increased mass, high cuboidal to columnar epithelium, increased size of follicles, and scanty colloid. In the other four freshwater fish (from Port John) the picture is essentially the same as that of the chum and this can be classed as quiescent to slightly active. An explanation of these phenomena is offered later.

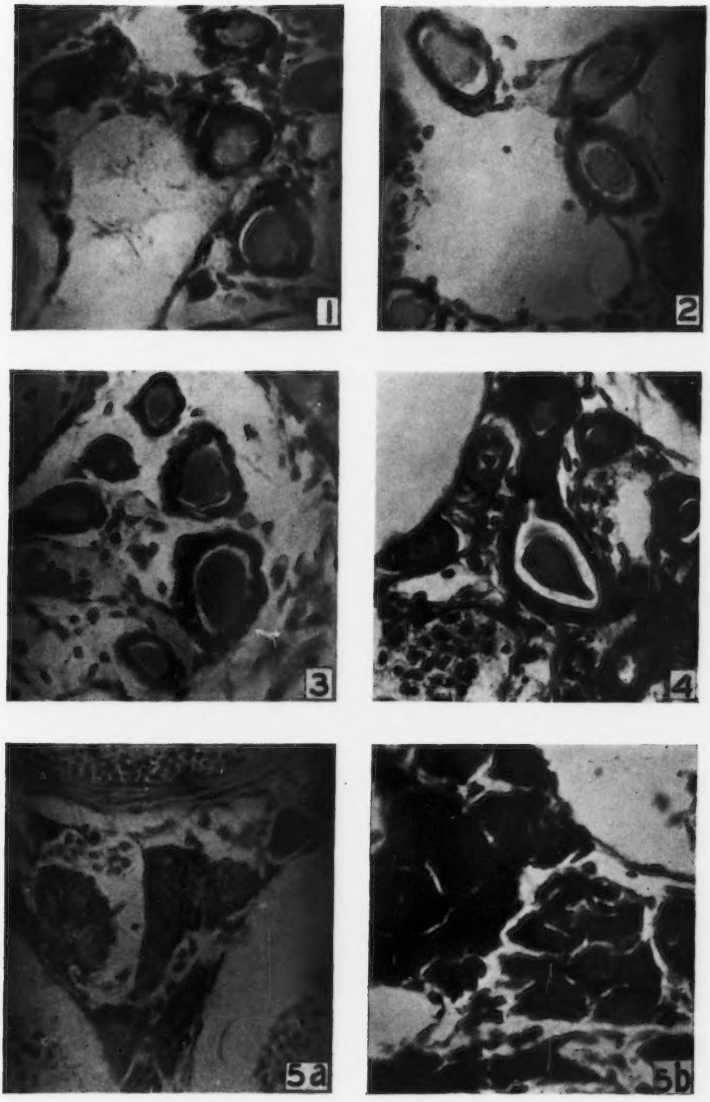
*Sockeye Fry*

The condition of the thyroid is uniform in the three individuals examined. The size of the organ is the smallest in the series (Fig. 6, Table II). The gland is quiescent. The follicles are small, the epithelium low, and the colloid acidophilic. Judged in a quantitative way, the activity is less than in chums and pinks. The specimens examined were migrating from stream to lake when captured.

*Coho Fry*

In the three coho fry the thyroid is scattered over a wider area than in the sockeye (Fig. 6) but the physiological condition as judged from the histology is the same.

PLATE I




*Photomicrographs of transverse sections of the thyroid glands of Pacific salmon (× 370).* FIG. 1. *Section of 39 mm. chum salmon from Nile Creek, Apr. 27, 1948.* FIG. 2. *Section of 42 mm. chum salmon from the sea at Port John, May 10, 1948.* FIG. 3. *Section of 44 mm. coho salmon from Keighley creek, July 20, 1949.* FIG. 4. *Section of 67 mm. chum salmon from the sea at Retreat Cove, June 5, 1948.* FIG. 5. *Section of 65 mm. chum salmon retained in Cowichan Lake Hatchery for 65 days; preserved Aug. 7, 1948; Sections "a" and "b" through two different levels; note absence of colloid and follicles converted to masses of epithelial cells.*

*Spring Fry*

The thyroids of the two spring fry are relatively larger than those of the sockeye and coho (Fig. 6), but show no greater activity. The colloid is strongly acidophilic, the follicles very small, and the epithelium low cuboidal in nature. These fish may or may not have been migrating to sea when captured. Fraser (7) found that two-thirds of the spring salmon do go down to sea during their first year.

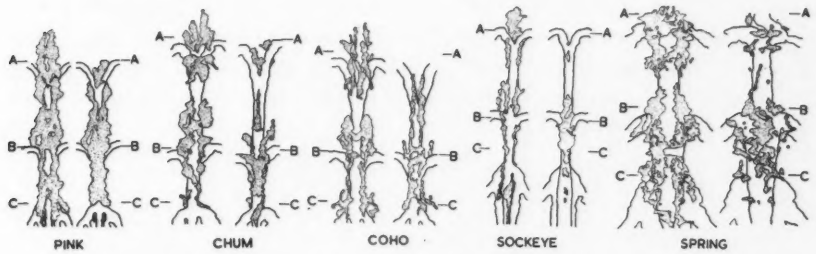



FIG. 6. *Graphic reconstruction (× 25) of the thyroid tissue (stippled) of Pacific salmon fry (30–40 mm. stage). In each case illustration on the left is the dorsal view and the illustration on the right is the ventral view.*

*Experimental**

Chum salmon fry become silvery during the alevin period and in this respect show a similar development to that of the Atlantic salmon or rainbow trout prior to migration. The silvering of *Salmo* has been attributed to the thyroid hormone (15, 22). If the deposition of guanine in the skin is dependent on thyroid hormone this phenomenon should be inhibited by treatment with antithyroid drugs.

Thirty chum salmon alevins, ranging in length from 30 to 35 mm. were maintained in a 0.36% solution of thiourea for 21 days. The same number of controls were held in tap water under identical conditions. The temperature was approximately 10° C. At the end of this period the yolk sacs of the controls were absorbed and the experiment was terminated.

Both groups developed a heavy coat of guanine crystals during this period. The controls were more heavily pigmented dorsally but no difference in silvering could be detected. Pronounced differences in growth did, however, occur. The instantaneous growth rate of the untreated group was 0.212 while that of the treated group was 0.153 for the experimental period. The yolk of the experimental fish was not completely used at the end of the period and a number of deformities were observed toward the end of the experiment. Histological examination of the thyroid revealed a hyperplastic gland charac-

* *These experimental data are taken from an unpublished thesis by S. B. Smith, Department of Zoology, University of British Columbia, 1949.*

teristic of the action of this drug (17, 19). The retardation in growth is similar to that observed for hybrid *Platypoecilus–Xiphophorus* (8).

*Under-yearling Stages (45-80 mm.)*

The fish considered in this group are the under-yearling coho, chum, and pink salmon during their first summer. Of particular interest are chum and pink fingerlings retained in fresh water for three months after their usual time of migration.

*Coho*

Three of the specimens (63-75 mm.) were collected from the Lower Cowichan river, on May 31, and retained for 65 days in the Cowichan Lake Hatchery. The four other fish examined (45 mm.) were collected from Keighley Creek on July 20.

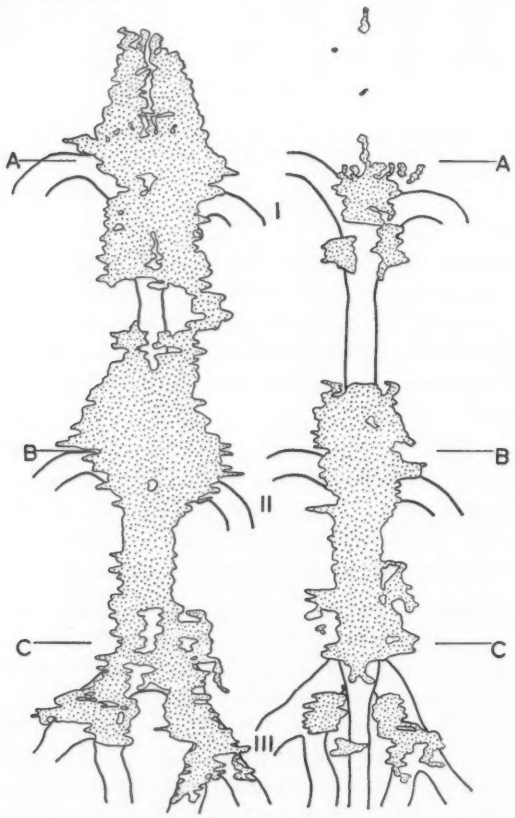



FIG. 7. *Graphic reconstruction (×25) of the thyroid gland (stippled) of a 63 mm. under-yearling coho salmon from Lake Cowichan Hatchery. Illustration on left is the dorsal view, that on the right the ventral view.*

As judged from the histology the thyroid of the coho during its summer in the stream is in a quiescent condition. The small spherical follicles are widely scattered and filled with acidophilic colloid. The epithelium is low cuboidal (Fig. 3). The distribution of the thyroid tissue of a 63 mm. coho is shown in Fig. 7.

*Chum—From Salt Water*

Four fish (48 mm., 67 mm., 80 mm., and 81 mm.) were examined from among specimens obtained in the sea at Retreat Cove on June 5, 1948. In all

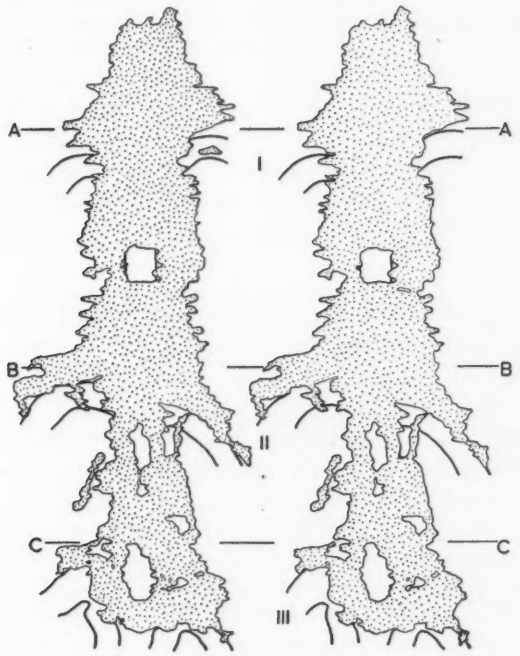



FIG. 8. *Graphic reconstruction (×25) of the thyroid gland (stippled) of a 65 mm. underyearling chum salmon from Lake Cowichan Hatchery. Illustration on the left is the dorsal view, that on the right the ventral view.*

fish the thyroid is quiescent (Fig. 4) and the picture the same as that of the chum at the time of seaward migration.

*Chum—Retained in Fresh Water*

Chum salmon were obtained from Lower Cowichan River on May 31, 1948, and retained in the hatchery at Lake Cowichan during the summer. Three specimens (53, 63, and 65 mm.) preserved on Aug. 7 have been examined. The thyroid picture is interesting.

The gland in these fish is in a stage of extreme hyperactivity (Fig. 5). The quantity of tissue is greatly increased (Fig. 8). The follicles are considerably enlarged and have become oval, irregular, or tufted in form. The epithelium is columnar with basal nuclei and lightly staining peripheral cytoplasm. The colloid is withdrawn and the follicular lumen almost obliterated. Activity, as judged from the histological picture, is the most extreme observed in salmon.

*Pink*

In pink salmon fingerlings the picture is identical with that just described for the chums. Specimens taken in the sea at Retreat Cove show quiescent to mildly active thyroids while those retained at Cultus Lake in 1934 until Aug. 15 (three specimens examined) have exceedingly hyperplastic glands.

*Yearling and Older Migrants*

The fish considered in this group are the seaward migrants of sockeye and coho salmon. The fish vary in age from one to two years and in size from 75 mm. to 175 mm. Spring salmon migrants in this size group are rare in the northern rivers and none was available for study.

*Sockeye Migrants*

Three sockeye migrants from Fort Babine have been examined. Two were one-winter fish, 91 mm. and 96 mm. respectively, preserved on June 6, 1948, and one was a two-winter fish, 138 mm. preserved May 30, 1948.

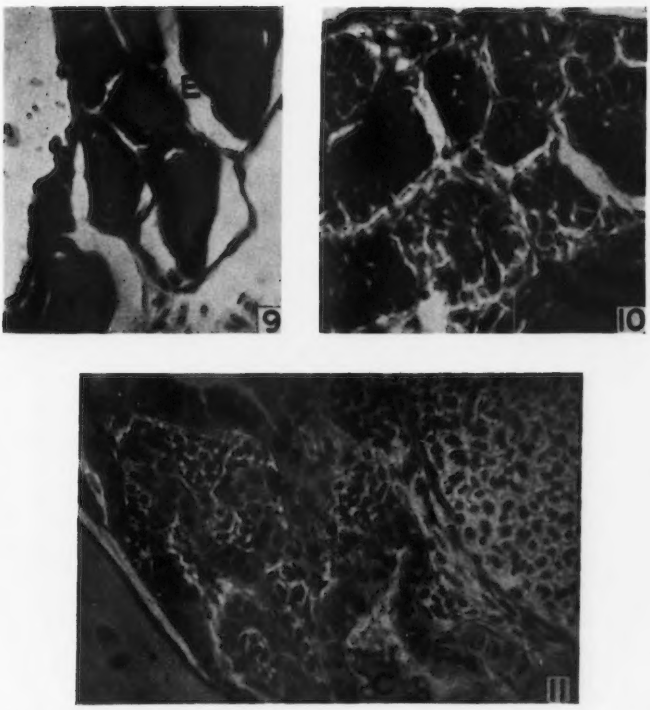
The thyroid of the migrating sockeye is relatively large and distributed over a wide area. Activity is variable in different sections but the anterior portion in all three migrants shows increased secretory activity. This is particularly pronounced in the 138 mm. specimen. Here follicular colloid is absent or, if present, riddled with peripheral vacuoles. The follicles are large, the epithelial walls irregular, and, in many, the vacuoles are tufted. The epithelial cells are high cubic or columnar (Fig. 11). Secretory activity, although less pronounced in the 91 and 96 mm. fish than in the 138 mm. specimen, is in all specimens definite in contrast to the 30-40 mm. fish.

*Coho Migrants*

Two yearling coho from Port John (74 mm. and 100 mm.) and five yearling coho from Fort Babine (143 to 160 mm.) were examined.

The picture is somewhat variable. The thyroids of specimens collected at Port John, May 2, 1948, are apparently only slightly more active than those of under-yearling fish. This might be attributed to the heightening in activity which occurs in many fish in the spring (11). There is an increase in the size of the follicles of the gland which may be somewhat irregular in shape but always contain abundant acidophilic colloid. The epithelium is never higher than cuboidal (Fig. 9).

PLATE II




*Photomicrographs of transverse sections of the thyroid glands of Pacific salmon (× 370). C, colloid; E, epithelium.* FIG. 9. *Section of 100 mm. coho migrant from Port John, May 2, 1948. Note the abundant acidophilic colloid and low epithelium.* FIG. 10. *Section of 146 mm. coho migrant from Fort Babine, June 6, 1948. Note scanty colloid and higher epithelium.* FIG. 11. *Section of 138 mm. sockeye migrant from Fort Babine, May 30, 1948. Note the columnar epithelium, irregular follicles, and vacuolated colloid.*

The Fort Babine specimens, collected June 6, 1948, show varying degrees of increased activity. In one specimen this is only slightly greater than that of the Port John fish. In the other specimens, however, the epithelium is higher, varying from high cuboidal to columnar, and there is evidence of withdrawal of the colloid. The follicles are either empty or contain lightly staining basic material with numerous peripheral vacuoles (Fig. 10).

## Discussion

It may be assumed that migratory behavior is dependent upon both external and internal environmental factors. The probable importance of internal environmental changes in relation to fish migration has been emphasized by Fontaine (3, 4, 5) and Koch (13). If thyroid activity is related to migration it may be expected to alter behavior patterns and (or) alter metabolism in such a way that the animal can more effectively deal with its new environment. So far there is little evidence to show that the thyroid hormone is related either to the behavior or the metabolism of fish migration. Findings pertinent to this discussion may be summarized briefly. Harms (9), by the use of thyroid materials, was able to cause premature development of the amphibian-like behavior in the goby *Periophthalmus*. More strikingly, he was able to induce the blenny, *Blennius ocellatus*, which normally spends its entire life in water, to assume a semiterrestrial existence by thyroid treatment. On the other hand, attempts to establish a relationship between thyroid activity and changes in the rheotactic responses of elvers have led to conflicting conclusions (5, 23). Studies of the thyroid hormone in relation to the metabolism of chlorides are suggestive. This work has shown that feeding thyroid hormone will produce definite changes in chloride metabolism of sticklebacks (10, 14) while changing the salinity of the environment evidently alters thyroid activity in several teleosts (16, 20). Thus, it would appear that the thyroid hormone plays a part in osmotic regulation and chloride balance in fish and might, in this way, be involved in the migration of anadromous or catadromous fish. Perhaps the most pertinent observation in this connection is that of Fontaine and Callamand (6). These workers, studying blood chlorides, have demonstrated that the smolt is much more sensitive to factors which disturb osmotic regulation than is the parr. The blood chlorides do not differ greatly in parr and smolt unless the fish are disturbed. Transportation of fish to the laboratory, which may possibly injure the more delicate skin of the smolt, produces profound changes in the chlorides of the migrating fish. The studies of the Pacific salmon thyroids in relation to their migratory behavior provide some further suggestive evidence.

At first the picture displayed by the thyroids of Pacific salmon is confusing. Migrating chum and sockeye fry have quiescent glands while the older sockeye migrants and the coho migrants usually have rather active glands. The activity of the pink salmon thyroid seems to vary from place to place. A seasonal change in the thyroid activity of salmon has been observed (11). In part the variations described for Pacific salmon may be purely seasonal. For

example, the mild activity of some of the sockeye or the coho migrants from Port John may be produced in this way. However, a different explanation must be sought for the more pronounced activity of the Fort Babine pink and coho migrants, for the majority of the older sockeye migrants, and particularly, for the chum and pink salmon retained in fresh water. The latter group suggests the most likely explanation.

Chum salmon retained in fresh water have been carefully studied (1, 2). It is evident that these fish are poorly adapted to this environment and will only survive under most favorable conditions. The Japanese workers found high mortalities and the Cowichan Lake Hatchery fish, from which our samples were drawn, suffered almost complete mortality toward the end of their first summer (1). Black (1) compared the tissue chlorides, water content, and density of under-yearling chums with coho of the same age. When placed in sea water the coho accumulate chloride until death while the chums are able to eliminate the chloride and survive. This is not surprising in view of the differences in life history of the two species. Of particular interest is the fact that coho, adapted to fresh water and not to sea water, have quiescent thyroids in their natural fresh water environments, while chum salmon likewise have quiescent glands in their natural environment, the sea, but have hyperplastic thyroid glands when held in the fresh water. Pink salmon evidently behave in the same manner as chums. Figures and tables already referred to show that chums and pinks have hyperplastic thyroids and this activity is extreme in relation to coho of the same size retained under identical conditions or chums and pinks which have been permitted to enter salt water.

The following is suggested as an explanation for the somewhat varied picture of the Pacific salmon thyroid gland. It may be assumed that these salmon, after the "smolt" transformation, are no longer completely adapted physiologically to fresh water. Further, the burden of osmotic regulation in a medium not entirely suitable can be met by increased production of thyroid hormone. The work of Olivereau (20) and Leloup (16) may be referred to in support of this last statement. These workers found increased production of thyroid hormone when the osmotic content of the environment was changed abnormally. This seems to be the most likely factor influencing the thyroid of these young Pacific salmon. Thus, the chum salmon normally spawn in the lower stretches of the rivers and go to sea as soon as they emerge from the gravel. The thyroid is well developed but shows no evidence of heightened physiological activity at the time of migration. If, however, the fish are retained in fresh water the thyroid becomes hyperactive. In general, the habits of pink salmon are the same but spawning may occur much farther up the rivers. The pink fry from Fort Babine in the headwaters of the Skeena river have more active thyroids than those at Port John emerging from the gravel beds near the river's mouth. Sockeye, coho, and spring salmon spend varying periods in fresh water and evidently can maintain their adjustments to fresh water for considerable to indefinite lengths of time. Sockeye fry migrating into the lake from the tributary stream have a thyroid that is

quiescent. On the other hand, the yearlings and older fish moving toward the sea show more active glands. This activity is probably due, in part, to a seasonal increase which has been observed in other species of fish but in part, too, to a physiological change related to the fresh water – salt water adjustment. The coho migrants are interesting in that those taken at Port John in May show much less activity of the thyroid than those taken at Fort Babine in June.

It is possible that the heightened activity described in Atlantic salmon (11) and steelhead trout (21) is likewise due to a stimulation of the gland by the increased metabolic work of osmoregulation in a fish prepared for life in salt water.

Finally, it should be pointed out that these findings do not establish a specific role for the thyroid hormone of fish. It is evident that this hormone is involved in many metabolic processes and osmotic regulation is only one of several chains of reactions in which it is important. The hormone is also important in growth as shown by the decreased growth rate of chum fry in thiourea solutions. Again, several workers have pointed out that thyroid treatment of salmonoids brings about increased utilization of fats (12). The deposition of guanine in the skin of the salmon might also be mentioned as a process stimulated by this hormone but not specifically controlled by it. Thyroid treatment evidently stimulates the production of guanine in salmon and trout (15, 22) but the silvering is rarely as pronounced as that which occurs under natural conditions (12) and thiourea does not interfere with the guanine production in chum salmon fry. The thyroid hormone of fish should be looked upon as a link in some chain of chemical reactions which is involved in a number of different metabolic processes.

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# DEPOSITION OF $P^{32}$ IN DEVELOPING *DROSOPHILA*[1]

By R. L. B. Irwin,[2] J. W. T. Spinks,[3] and T. J. Arnason[4]

## Abstract

Eggs of Canton Special stock of *Drosophila* were hatched and larvae reared on medium containing $P^{32}$. Alternate radioautographed and stained sections were made of larvae, pupae, and adults. Deposition of $P^{32}$ was found to be heavy in fat bodies, nervous structures, and in organs of digestion and excretion. Very high concentrations of $P^{32}$ occurred in imaginal discs. Discharged materials and nuclei from fat bodies had much $P^{32}$ in early pupal stages, the activity becoming concentrated at sites of organ differentiation in later pupae. In the adults, the gonads, the nervous system, and the digestive tract contained much $P^{32}$.

## Introduction

Radioactive phosphorus, incorporated in the food supplied to *Drosophila* larvae is lethal (preventing emergence) if at an initial concentration of 1.3 rd. per ml. food (2). It is also observed that pupation is delayed or completely inhibited by the ingested radioisotope and that mortality in the pupal stage is high when high concentrations of $P^{32}$ are used. It was thought that an investigation of $P^{32}$ distribution in larvae and pupae might be of value in determining how the lethal effects are brought about. At the same time information on differential $P^{32}$ accumulation and dispersal might provide information relevant to certain problems in developmental genetics.

## Materials and Methods

The Canton Special wild strain of *Drosophila melanogaster* was used in this investigation. Eggs, collected within eight hours of laying, were transferred to 5 ml. vials containing 0.16 rd. $P^{32}$ in 1 ml. cornmeal molasses agar (9). The culture vials were then placed in an incubator designed to maintain high humidity. The temperature remained within two degrees of 25° C.

Individuals were collected for sectioning, four at a time, at the following ages: larvae at 2.5 and 5 days, pupae at 6 and 9 days, and adults, as they emerged, at 13 days.

Larvae, pupae, and etherized adults were killed and fixed in boiling water, dehydrated in absolute alcohol, and embedded in Parlax. Cross sections were cut at 10$\mu$. Sections from each individual were numbered serially. Odd numbered sections were arranged in order on one set of slides for staining with Mallory's triple stain, even numbered sections were mounted serially on a second set of slides for radioautographing. Thus for each radioautographed section there is a closely corresponding pair of stained sections. The main anatomical references used were those of Lowne (6) and Snodgrass (10, 11, 12).

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Bélanger and Leblond's (3) method of autographing was followed. The method involved removing emulsion from Kodak medium lantern slides and transferring it to a beaker on a hot plate (40°–50° C.). A camel's hair brush was used to paint the emulsion over the *Drosophila* sections, which had been treated previously with a drop of 1% celloidin. The estimated thickness of the emulsion was 6 to 10$\mu$. Emulsion-coated slides were stored in a dark cupboard for 360 hr., then developed, dried, and cover slips attached by means of Canada balsam.

Determination of relative amounts of $P^{32}$ in different structures was facilitated by detailed comparison of the closely corresponding sections on the two sets of slides (see e.g. Figs. 7–12). Photomicrographs of selected sections were taken. Semidiagrammatic camera-lucida drawings have also been prepared as an aid to interpretation.

## Removal of Radioactivity by Processing

The amounts of radioactivity transferred from each of several individuals to the solutions used in fixing, dehydrating, and embedding were determined. The average percentage of $P^{32}$ lost to each solution is recorded in Table I.

TABLE I

AVERAGE PERCENTAGE OF RADIOACTIVITY LOST IN WATER, ETHYL ALCOHOL, XYLOL, AND PARLAX-XYLOL 1 : 1 IN PROCESS OF EMBEDDING

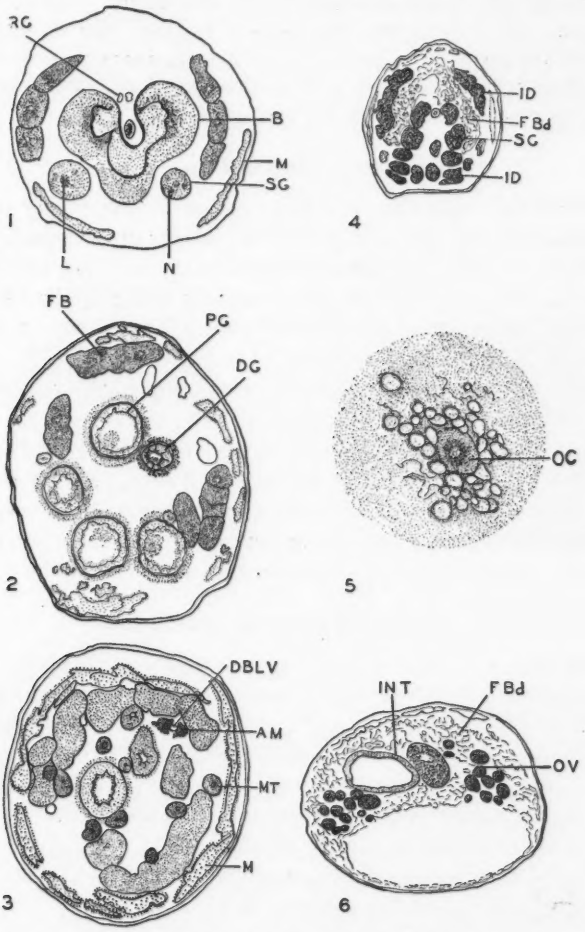
| Age, days | Stage | No. tested | % $P^{32}$ lost in water (5 min.) | % $P^{32}$ lost in alcohol (8 hr.) | % $P^{32}$ lost in xylol (8 hr.) | % $P^{32}$ lost in parlax–xylol 1 : 1 (8 hr.) |
|---|---|---|---|---|---|---|
| 5 | 5 day larva | 6 | 11.9 | 22.06 | Negligible | Negligible |
| 6 | 1 day pupa | 5 | 8.1 | 11.6 | " | " |
| 11 | 5 day pupa | 5 | 7.5 | 3.8 | " | " |

With advancing age there is a decrease in the amount of $P^{32}$ lost to water and a striking decrease in the amount of $P^{32}$ lost to alcohol. Xylol and 1 : 1 xylol and parlax removed a negligible amount of radioactivity.

## $P^{32}$ Distribution in Larvae

Less $P^{32}$ was deposited in the central stromal tissue of the brain than in the outer cortex. Drawings of a section cut through the posterior part of the brain (Fig. 1) and of one of the supraoesophageal ganglia (Fig. 15) illustrate this unequal distribution. Posterior to the brain, differential deposition of phosphorus was seen wherever stromal and cortical tissue could be distinguished (e.g. Fig. 13, NC). The ring gland, studied in conjunction with the brain, appeared to contain a very small amount of the radioactive material (Fig. 1, RG).

Of the organs associated with digestion and excretion the salivary glands and the malpighian tubules (Fig. 3, MT) showed particularly heavy $P^{32}$ content. Considerable phosphorus occurred in the cells and lumen of the proventriculus and along both inner and outer surfaces of the mid-gut distal to the flexure (Fig. 2, PG, DG). Little phosphorus was found in the rectum.




FIGS. 1 – 6. *Camera lucida drawings based on radioautographs. Semidiagrammatic.*

FIG. 1. *Cross section of 5-day larva. B—brain, M—muscle, SG—salivary gland, N—nucleus, L—lacuna, RG—ring gland.* ×100. FIG. 2. *Cross section of 5-day larva. FB—fat body, PG—proximal part of mid-gut, DG—distal part of mid-gut.* ×70. FIG. 3. *Cross section of 5-day larva. DBLV—dorsal blood vessel, AM—alary muscle, MT—malpighian tubule,* ×70. FIG. 4. *Cross section of 5-day larva. ID—imaginal disc, F Bd—fat body disintegrating.* ×70. FIG. 5. *Pupa at 6 days. Detail of small portion. Fat-cell nucleus (OC) surrounded by discharged fat-body contents.* ×700. FIG. 6. *Cross section of adult female. INT—intestine, OV—ovary.* ×70.

Larval muscles showed $P^{32}$ lightly distributed except for the alary muscle which had a heavy $P^{32}$ content (Fig. 3, M, AM). Imaginal discs in mature larvae contained much phosphorus (Fig. 4, ID). In the young larvae a heavy accumulation of phosphorus occurred in the fat bodies. The anterior part of the fat body had a higher $P^{32}$ content than did the posterior part. In old larvae $P^{32}$ concentration decreased considerably in this organ (Fig. 2, F B and Fig. 4, F Bd). A study of the large cells of the fat body showed high $P^{32}$ content in the reticulate cytoplasm with the highest concentration around the nucleus. There seemed to be a more concentrated deposition around the nuclear periphery than within the nucleus itself (Fig. 16).

## $P^{32}$ Distribution in Pupae

Gross differential deposition was not obvious in the young developing pupae. Breakdown and dispersal of the cell contents of the anterior portions of the fat body may have contributed largely to the observed relatively even distribution of phosphorus as seen, e.g. in sections of the middle region of the pupae. Under high magnification (1000×) the $P^{32}$ was found to be concentrated in many separate small clusters. The position of the clusters in the radioautographs corresponds to that of nuclei surrounded by globules from the disintegrating cytoplasm of fat cells (Fig. 5).

In old pupae the developing organs are approaching the adult condition. The deposition of $P^{32}$ is heaviest in the regions of rapid growth and differentiation (Fig. 13). Examples of the increase in density of the phosphorus were seen especially in developing muscles, leg and wing discs, the developing optic stalks from the brain, and developing germinal epithelium of the ovaries and testes.

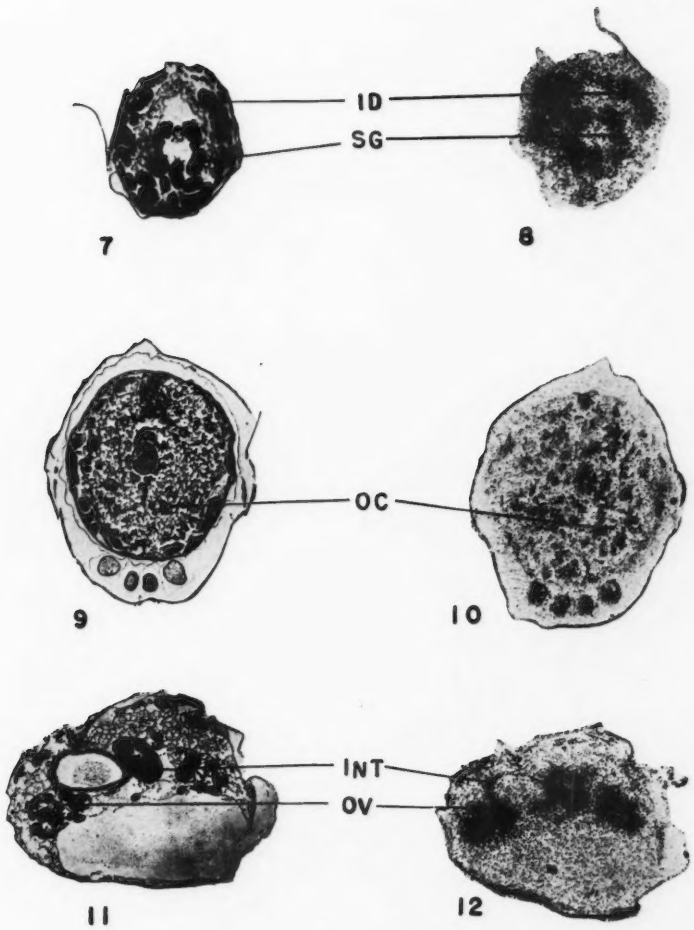
## $P^{32}$ Distribution in Adults

The major sites of heavy $P^{32}$ deposition are clearly outlined in radioautographs of sectioned adults. The brain and nerve cord, the optic stalks and especially the retina contain much $P^{32}$. However, within the stroma of the brain, $P^{32}$ is rather lightly and evenly distributed except in fissure areas of the corpus centrale. The nerve cord continues to maintain a differential distribution, heavy in the cortical, light in the stromal area (Fig. 14).

Other structures containing much $P^{32}$ include the following: the mesothoracic muscles, oesophagus and the oesophageal valve, the mid and hind gut, the malpighian tubules, and the gonads (Fig. 6, OV).

## Discussion

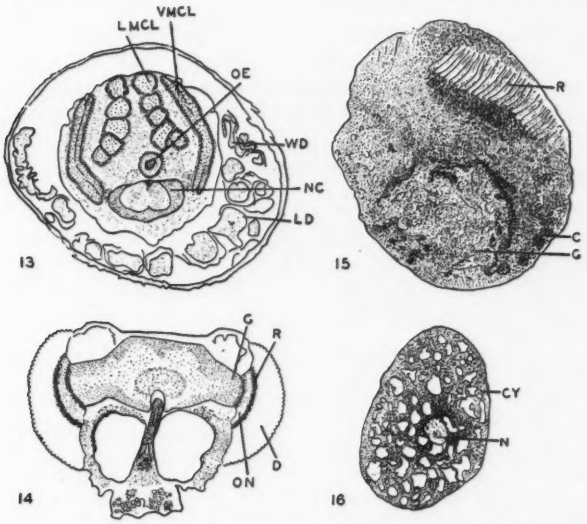
The phosphorus remaining in the tissues after passage through the fluids used in fixing and embedding must be largely in nucleoproteins, nucleic acids, and other large organic molecules. To judge by the amounts of $P^{32}$ passing into the various solutions, a surprisingly large proportion of the contained phosphorus is in insoluble form, particularly in pupae.




FIGS. 7 – 12. *Stained and radioautographed sections compared.*

FIG. 7. *Photomicrograph of stained section, 5-day larva, compare with Fig. 8, radioautograph and Fig. 4, drawing.* ×70. FIGS. 9 AND 10. *Adjacent sections from 8-day pupa. Stain cf. radioautograph. Note scattered distribution of* $P^{32}$ *in the radioautograph.* ×60. FIGS. 11 AND 12. *Adjacent sections of adult stained and radioautographed.* ×75. *Compare with drawing. Fig. 6.*

Earlier investigations, e.g., that of Marshak (7), have shown that phosphorus tends to become concentrated in nuclei of dividing cells. A high content and rapid turnover of ribose nucleic acids, rich in phosphorus, occurs




**FIGS. 13 – 16.** *Semidiagrammatic camera lucida drawings based on radioautographs.*

FIG. 13. *Pupa, 9 days, cross section. LMCL—longitudinal muscle, VMCL—vertical muscle, OE—oesophagus, WD—wing disc, NC—nerve cord, LD—leg disc.* ×65. FIG. 14. *Adult, 14 days, cross section, head region. G—ganglion matrix, R—retina, D—dioptron, ON—optic stalk.* ×65. FIG. 15. *Single supraoesophageal ganglion from 5-day larva. R—retina, C—cortex, G—matrix of ganglion.* ×230. FIG. 16. *Fat-body cell from 5-day larva. CY—cytoplasm, N—nucleus.* ×140.

in the cytoplasm of metabolically active, e.g., growing, secreting cells (5). Protein-formation is promoted by nucleic acids according to Caspersson (4).

When concentrated in actively growing or secreting cells of immature individuals $P^{32}$ is in position to emit beta particles with possible damaging effects since slight alterations in early growth processes may cause large deviations from the normal condition at later stages. Inclusion of $P^{32}$ in gene and enzyme molecules doubtless contributes to the effectiveness of the isotope. Since there is virtual absence of cell division in larvae within a day after hatching (8), one may infer that heavy deposits of $P^{32}$ in larvae reflect active metabolism, apart perhaps from the surfaces which are in direct contact with the radioactive food whose apparent content may result from $P^{32}$ adsorption. Thus, salivary glands and malpighian tubules have high $P^{32}$ content in both young and old larvae that have fed on a medium containing inorganic $P^{32}$.

It is noteworthy that in young larvae high $P^{32}$ concentration occurs in the fat bodies, with some indications of a gradient in individual cells, the highest concentration being at the nuclear membrane. In old larvae there is a significant reduction in $P^{32}$ content of fat bodies and a conspicuous concentration

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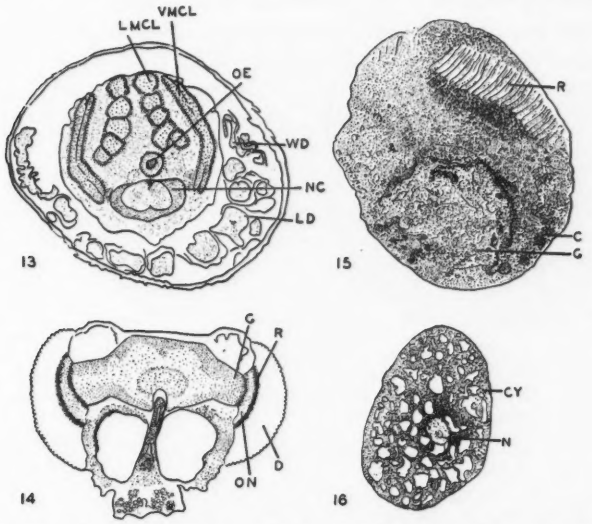


**FIGS. 13 – 16.** *Semidiagrammatic camera lucida drawings based on radioautographs.*

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in the imaginal discs. After feeding stops (pupa) the nuclei of fat cells surrounded by cytoplasmic debris are also rich in phosphorus. The high phosphorus content of the cells of the fat body may, in $P^{32}$-treated individuals interfere with their efficient functioning.

In old pupae and in adults, when general growth has almost or quite ceased, the gonads continue to show very high $P^{32}$ content. This fact is of some genetical interest, since concentration of $P^{32}$ in testes and ovaries must increase the likelihood of induced mutations in germ cells of individuals treated in this way (1).

## Acknowledgments

We wish to acknowledge financial assistance from the National Research Council of Canada. We are grateful also to Dr. L. G. Saunders, Dr. J. G. Rempel, and Dr. R. Altschul for many helpful suggestions.

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# THE FATE OF PHENOTHIAZINE IN THE GASTROINTESTINAL TRACT OF THE SHEEP[1, 2]

By R. P. Harpur,[3] W. E. Swales,[4] and O. F. Denstedt[5]

## Abstract

The methods for estimating phenothiazine and phenothiazone in biological materials have been investigated. It has been shown that when phenothiazine, or phenothiazone is treated with bromine (under the conditions described), a red color is produced which has absorption maxima at the following wave lengths: 240, 282, 291, 375, and 510 mμ. Phenothiazone, in 95% ethanol, has a spectrum with absorption maxima at 237, 273, 280, 288, 369, and 508 mμ. Thus the bromine method is not applicable for distinguishing between these two compounds. It is shown also that phenothiazine may interfere with the estimation of phenothiazone when the latter is measured by the light absorption of its free radical. The ultraviolet absorption spectra of phenothiazine and diphenylamine *o*-sulphoxide have been determined. The spectrum of the former compound has a maximum at 254 mμ (with a small maximum at 318 mμ), and that of the latter has maxima at 227, 272, 302, and 335 mμ. Leucophenothiazone also has a maximum at 254 mμ.

While excretion studies on sheep confirm that 40% of the orally administered phenothiazine is lost in the urine, it is shown that the absorption of the drug from the intestine is not dependent upon oxidation to phenothiazone in the rumen. Indeed, the drug appears to be unchanged in this organ. In a post-mortem examination of the gastrointestinal tract no phenothiazone was found proximal to the bile duct. It is suggested that phenothiazine is absorbed from the rumen unoxidized. In support of this it is confirmed that some phenothiazine may be recovered unchanged from the urine of the sheep.

## Introduction

Phenothiazine (thiodiphenylamine) is an effective agent for the removal of intestinal worms and in recent years has come into widespread use for the treatment of sheep. The present investigation, which is an extension of the studies of Collier and Swales (5, 6, 23), concerns the metabolism of phenothiazine in the sheep, with particular reference to the manner in which the drug is modified at various points in the intestinal tract.

The dose required for full anthelmintic activity in the sheep is 0.3 to 0.5 gm. per pound body weight (8). Not only is this a large and expensive dose but, as Swales and Collier (23) have shown, about 40–50% of the drug, in the form of oxidized derivatives, is lost in the urine. Two questions arise: first, is a high concentration of phenothiazine or a derivative necessary for anthelmintic activity, and, second, what are the factors which influence the absorption of the drug from the intestine? If more were known about these problems, it might be possible to decrease the dosage and hence the cost of medication.

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*Contribution from the Institute of Parasitology, Macdonald College, P.Q., and the Department of Biochemistry, McGill University, Montreal, P.Q., with financial assistance from the National Research Council of Canada.*

[2] *From the M.Sc. Thesis of R. P. Harpur, McGill University, 1947.*

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Furthermore, while phenothiazine is relatively nontoxic for the sheep, it may be harmful to other animals, hence a wider knowledge of its pharmacology is desirable.

When ruminants are dosed with phenothiazine a strong anthelmintic action occurs immediately posterior to the rumen. In monogastric herbivores, which have a comparatively large caecum to replace, in part, the digestive functions of the rumen, the most efficient anthelmintic action appears to take place in, and posterior to, the caecum. In the horse, for example, the anthelmintic activity anterior to the caecum is weak.

In carnivores, in which there is no organ analogous to the large active rumen or caecum of the herbivores, phenothiazine is not anthelmintic even to hookworms (Ancylostomidae), which are easily destroyed by this chemical in ruminants. A detailed study of the fate of phenothiazine in the rumen of the sheep, therefore, is necessary.

The fate of phenothiazine in the animal body has been studied in several species. Eddy *et al.* (12) reported that the metabolites excreted in the urine of the rat, rabbit, and human are thionol, leucothionol, and unidentified conjugates of phenothiazine and leucothionol. Lipson (15) demonstrated the presence of phenothiazine, thionol, and phenothiazone in the acidified urine of the sheep. Collier (5) identified phenothiazine in the faeces of sheep (later phenothiazone also (6) ) and an ethereal sulphate of leucophenothiazone in the urine, blood serum, and milk. Later, Collier and his associates (6) presented strong evidence for the presence and identity of the sulphuric acid conjugate. Using Lipson's chromatographic method, they showed that the pigment in sheep urine consists almost entirely of phenothiazone with a trace of thionol.

De Eds and Thomas (10) found oxidation products of phenothiazine in the bile of the rabbit, dog, and man. Similarly, Collier *et al.* (6) demonstrated the presence of the conjugate in the bladder bile of a sheep five hours after dosing with 100 gm. of phenothiazine. The rumen of the same animal contained 58 gm. of unchanged phenothiazine and about 4 gm. of phenothiazone. The conclusion drawn was that phenothiazine undergoes rapid oxidation in the rumen. The prompt appearance of phenothiazone in the blood and urine was attributed to the increased solubility and ready absorption of this derivative. Clare (4) was unable to find phenothiazone in the rumen, but has reported the presence of diphenylamine *o*-sulphoxide in the rumen of sheep after dosing with phenothiazine. It is clear, therefore, that the fate of the ingested drug may be variable.

In our studies the sulphoxide was not found in the rumen but the observations reported in this paper confirm the finding of Clare (4) that phenothiazine normally is not oxidized to phenothiazone in the rumen. In addition we have been able to confirm the observation of Lipson (15) that phenothiazine is present in the acidified urine of sheep after dosing with this drug.

## Preparation of Materials

*1. Phenothiazine*

Commercial phenothiazine was purified by steam distillation (1), followed by recrystallization twice from 95% ethanol. The product finally was washed with carbon tetrachloride and dried. The crystals melted at 185° C. (19).*

The solubility of phenothiazine in water has been reported as 1 part in 800,000 (8). We confirmed this in the following manner:

An excess of phenothiazine was added to a large volume of freshly boiled distilled water. This was protected from direct light and kept at the desired temperature for about three hours with frequent stirring. The solution was then extracted twice with chloroform, the extract evaporated to dryness, the residue taken up in 95% ethanol, and phenothiazine estimated by the red color produced on the addition of bromine.

The results are given in Table I:

TABLE I

SOLUBILITY OF PHENOTHIAZINE

| Temperature (centigrade) | Solubility (gm. per 100 ml. water) |
|---|---|
| 20° | 0.00010 |
| 23° | 0.00014 |
| 39° | 0.00024 |
| 60° | 0.00063 |

*2. Diphenylamine o-sulphoxide*

This compound was prepared by a modification (18) of the method of Barnett and Smiles (2). The product melted at 250° C.

*3. Phenothiazone*

Phenothiazone was prepared from phenothiazine by the method of Pummerer and Gassner (18). The crude product was recrystallized from water, and then from 95% ethanol to give microscopic brick-red crystals which melted at 161° C. (13).

*4. Leucophenothiazone*

The leuco-compound was prepared by the following procedure (3):

A small amount of phenothiazine (0.25 gm.) was covered with 5 ml. of 95% ethanol, and stannous chloride (0.5 gm.) and concentrated hydrochloric acid (0.25 ml.) were added. On heating, the phenothiazine and stannous chloride dissolved and the solution turned green, then colorless. On pouring the solution into 20 ml. of cold water, a white precipitate of leucophenothiazone

** All melting points were determined by means of a hot-stage microscope equipped with a thermocouple.*

was obtained. After centrifuging, the supernatant liquid was decanted, the precipitate washed twice with cold acidified water, and dried *in vacuo* at 100° C. The product was recrystallized from chloroform, filtered in an atmosphere of carbon dioxide, and washed with cold petroleum ether.

Under the microscope sheaves of slender white crystals were seen, which, on heating to above 140° C., turned purple in color before melting. It was difficult to obtain a sharp melting point. The melting point of leucophenothiazone has been reported as 172–3° C. (11), but we have been unable to confirm this finding. With a temperature increment of less than one degree per minute, melting occurred over the range of 164–183° C. In an atmosphere of nitrogen, the range of melting was 179–183° C. It seems probable that leucophenothiazone is oxidized to phenothiazone on heating, and that the melting point of the product varies with the extent of oxidation.

## Extraction of Materials

*1. Phenothiazine*

In the extraction of the drug from faeces, the material was dried and ground, and then was extracted with hot 95% ethanol (5). The same method, when applied to rumen contents, gave low recoveries of the drug. When the extraction was carried out on wet material, however, the recovery was satisfactory. The method finally adopted was to centrifuge the rumen material and filter the supernatant fluid. The combined residues, after the centrifugation and filtration, were extracted several times with hot 95% ethanol.

*2. Phenothiazone*

This substance was extracted from the rumen contents with boiling water. Preliminary tests showed that, for complete recovery of phenothiazone from rumen contents, at least four extractions of the solid residue are required.

In all these extractions material from untreated animals was shown to be free from interfering substances.

## Methods of Analysis

*1. Colorimetric Methods*

Phenothiazine was estimated by the procedure of Collier (5) modified as recommended by Cupples (7). The method consists in adding bromine-water to a hot alcoholic solution of phenothiazine and measuring the intensity of the red color produced. Stewart (20) states that the transmission of the red color is minimum at 510 mμ. In the earlier part of our study a Klett-Summerson photoelectric colorimeter was used with a No. 54 filter, but later an Evelyn instrument was used with a 520 mμ filter.

Phenothiazone and leucophenothiazone in urine and aqueous extracts were estimated by a modification (24) of Collier's method (5). The method depends upon the absorption spectrum of the free radical of phenothiazone (16) which has a maximum absorption at 550 mμ. The free radical of phenothiazine has

a series of absorption bands, the one with the longest wave length having a maximum at 513 mμ. The latter, unfortunately, interferes with the corresponding band in the spectrum of the phenothiazone free radical even when readings are made with a 540 mμ filter. Thus, while the method is not specific for phenothiazone, it is satisfactory if the latter is the predominating derivative.

*2. Spectrophotometric Methods*

*Instruments*

The absorption spectra in both visible and ultraviolet regions were determined with a Beckman Quartz spectrophotometer, Model B. For qualitative examination of the spectra in the visible range a Hartridge reversion spectroscope was used. The latter instrument was calibrated against the prominent emission spectral lines of mercury and sodium. With the solutions, which we were analyzing, a larger width of slit was required than that used in the calibration. As only one jaw of the slit was movable, opening of the slit increased the width of the spectral line and shifted the mean position towards the longer wavelengths. A correction (−4 mμ), therefore, was made in all the readings taken with the Hartridge instrument.

*Oxidation with Bromine*

In our study, since it was necessary to identify and estimate various derivatives of phenothiazine, the effect of bromine treatment on phenothiazone in alcoholic solution was investigated. The absorption spectrum of phenothiazone (in 95% ethanol) was compared with that of bromine-treated phenothiazine and with that of bromine-treated phenothiazone. The absorption spectra are illustrated in Figs. 1 and 2. It will be seen that the spectra obtained when phenothiazine and phenothiazone are treated with bromine water are identical. Furthermore, the curves are similar to that of phenothiazone itself except for a slight bathochromic shift. It is apparent, therefore, that phenothiazine and phenothiazone cannot be distinguished by this method.

*Visual Absorption Spectra*

The spectra of the free radicals of phenothiazine and some of its derivatives in acid solution have been studied by Michaelis and his co-workers (13, 16, 17). Zukel (26) used the color reactions with concentrated sulphuric acid as a means of distinguishing phenothiazine and phenothiazone.

In Figs. 3, 4, and 5 are plotted the absorption spectra of pure samples of phenothiazine, phenothiazone, and diphenylamine sulphoxide. These substances were dissolved in concentrated sulphuric acid without the addition of oxidizing or reducing agent. As these conditions do not produce the maximum concentration of the free radicals, the molecular extinction coefficients are only of qualitative significance. However, they do serve as a guide for qualitative analysis with a visual spectroscope.

It will be noted in Fig. 3 that when the solution is allowed to stand, the phenothiazine spectrum changes to that characteristic of the sulphoxide. Also it will be noted in Figs. 3 and 5 that, on dilution of the solution with water, the

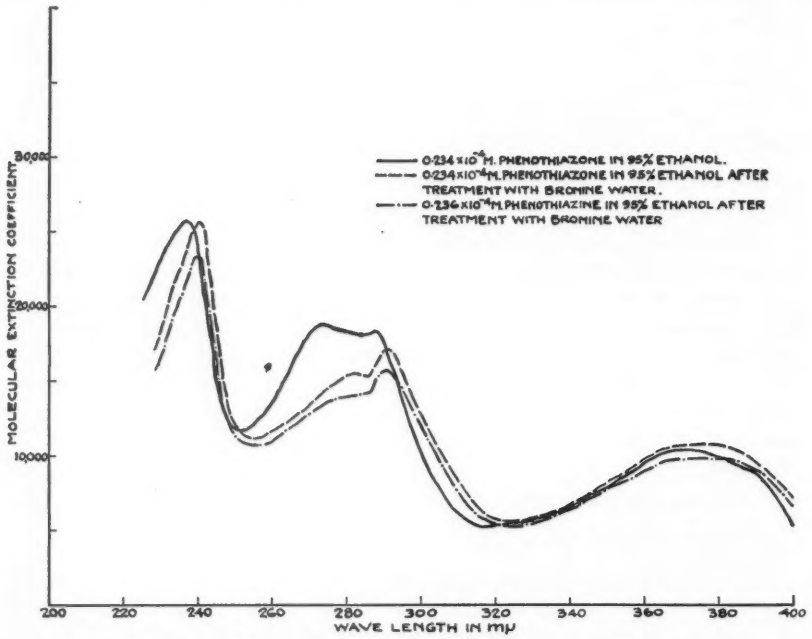


FIG. 1. *Comparison of the absorption spectrum of phenothiazone with that of phenothiazone and phenothiazine after treatment with bromine (ultraviolet).*

phenothiazine and sulphoxide spectra become qualitatively indistinguishable, so that, in the identification of these compounds, aqueous extracts must be taken to dryness before sulphuric acid is added. Under these conditions, phenothiazine gives an absorption band at 512–518 m$\mu$, phenothiazone at 502–504 m$\mu$, and the sulphoxide at 460–465 m$\mu$. On dilution of the solution with water the phenothiazine band remains unchanged, and that of the sulphoxide becomes indistinguishable from that of phenothiazine while the band of phenothiazone is shifted to the 540–560 m$\mu$ region.

*Ultraviolet Absorption Spectra**

Ethanol (95%) or chloroform was used as the solvent in the determination of ultraviolet absorption spectra. The curves of phenothiazine and diphenylamine *o*-sulphoxide are shown in Figs. 6 and 7. Preliminary experiments indicated that the ultraviolet absorption spectrum of leucophenothiazone is indistinguishable from that of phenothiazine. Diphenylamine *o*-sulphoxide, however, can be differentiated from the other compounds by this method.

The following procedure was developed for the examination of biological fluids. The sample (generally 10 ml.)was extracted with chloroform (10 ml., 10 ml., and 5 ml.) and the ultraviolet absorption spectrum of the extract was

* *Just before this paper was submitted for publication a paper by Houston et al. (14) appeared dealing with the ultraviolet absorption spectra of phenothiazine derivatives.*

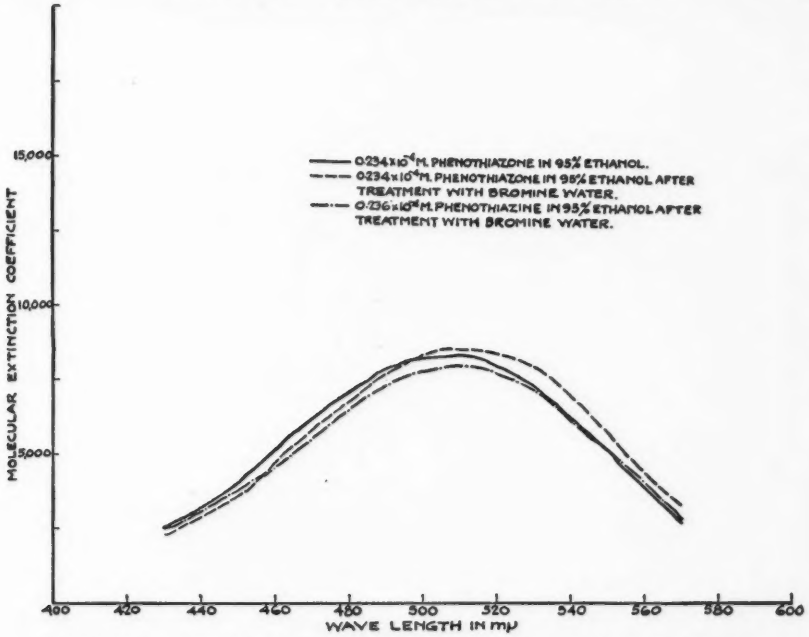
0·234×10⁻⁴M. PHENOTHIAZONE IN 95% ETHANOL.
0·234×10⁻⁴M. PHENOTHIAZONE IN 95% ETHANOL AFTER TREATMENT WITH BROMINE WATER.
0·236×10⁻⁴M. PHENOTHIAZINE IN 95% ETHANOL AFTER TREATMENT WITH BROMINE WATER.
MOLECULAR EXTINCTION COEFFICIENT
15,000
10,000
5,000
400 420 440 460 480 500 520 540 560 580 600
WAVE LENGTH IN mμ

FIG. 2. *Comparison of the absorption spectrum of phenothiazone with that of phenothiazine and phenothiazine after treatment with bromine (visible).*

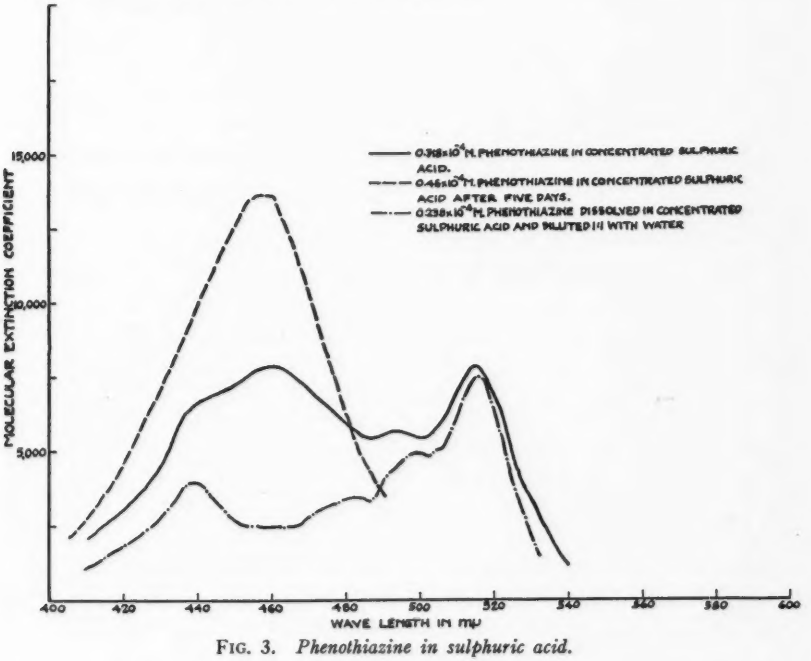
0·318×10⁻⁴M. PHENOTHIAZINE IN CONCENTRATED SULPHURIC ACID.
0·46×10⁻⁴M. PHENOTHIAZINE IN CONCENTRATED SULPHURIC ACID AFTER FIVE DAYS.
0·238×10⁻⁴M. PHENOTHIAZINE DISSOLVED IN CONCENTRATED SULPHURIC ACID AND DILUTED IN WITH WATER
MOLECULAR EXTINCTION COEFFICIENT
15,000
10,000
5,000
400 420 440 460 480 500 520 540 560 580 600
WAVE LENGTH IN mμ

FIG. 3. *Phenothiazine in sulphuric acid.*

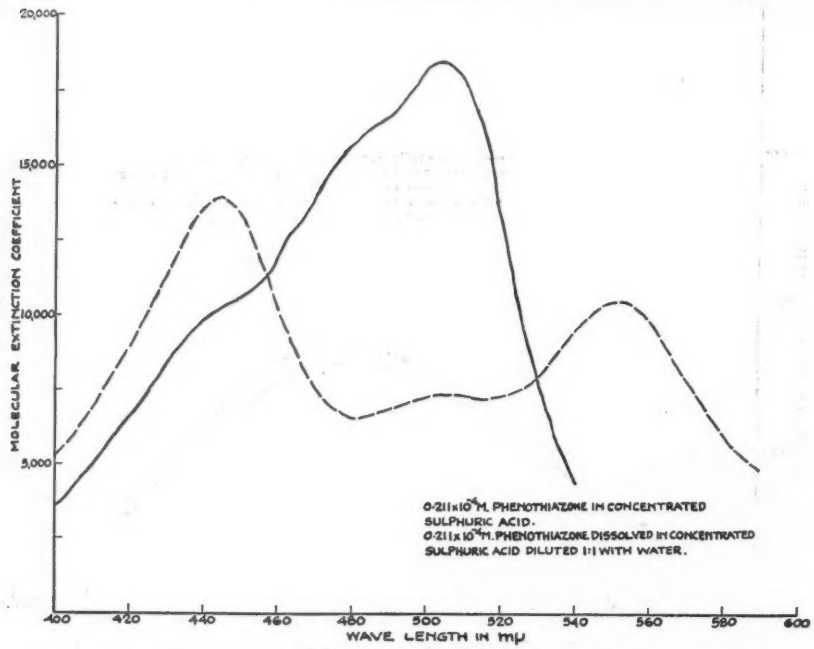


FIG. 4. *Phenothiazone in sulphuric acid.*

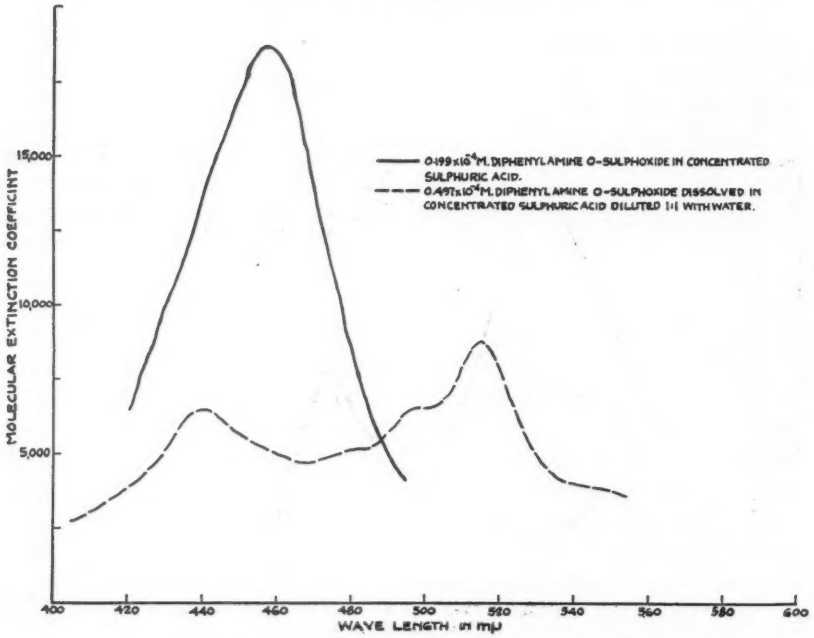


FIG. 5. *Diphenylamine o-sulphoxide in sulphuric acid.*

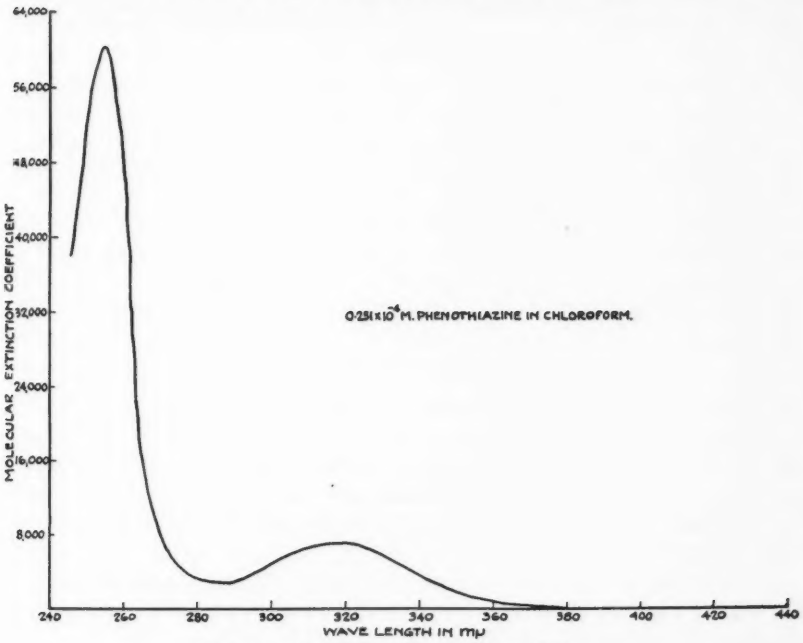


FIG. 6. *Absorption spectrum of phenothiazine.*

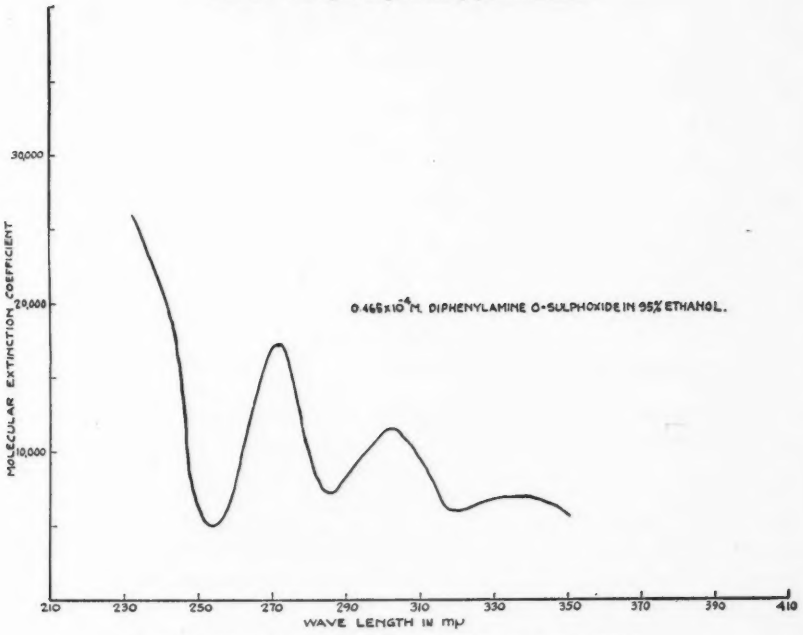


FIG. 7. *Absorption spectrum of diphenylamine o-sulphoxide.*

examined. A maximum at 254 mμ was taken to indicate the presence of phenothiazine and/or leucophenothiazone, while maxima at 272 and 304 mμ represented the presence of diphenylamine sulphoxide. The sample was then made strongly acid by addition of 4 ml. concentrated hydrochloric acid, and re-extracted with chloroform. An increase in the amount of phenothiazine or leucophenothiazone extracted from the acidified sample, over the amount extracted before acidification, was considered as evidence of the presence of either or both of these substances in the original extract in a conjugated form. The acidified sample was examined also with the Hartridge spectroscope. An absorption band at 540–560 mμ indicated the presence of phenothiazone, while a band at 512–518 mμ was indicative of phenothiazine or the sulphoxide.

## Excretion Studies

*Influence of Particle Size on Absorption of Phenothiazine*

Collier *et al.* (6) compared the anthelmintic activity, and the excretion in the urine, of micronized phenothiazine with that of the ordinary commercial product. To test further the effect of particle size, we compared the excretion of ordinary phenothiazine with that of a new commercial product of considerably larger particle size.

Four wethers, which may be designated A, B, C, and D, were dosed with 30 gm. of phenothiazine in tablet form (21). Sheep A and C were given the ordinary phenothiazine, and B and D, the coarser material. The excretion of phenothiazine and phenothiazone in the urine and faeces was followed for six days. The results obtained are similar to those obtained by Swales and Collier (23). Table II shows the total recoveries of the drug expressed as phenothiazine.

TABLE II

RECOVERY OF PHENOTHIAZINE

| Subject | Type of drug | Faeces | Urine* | Total |
|---|---|---|---|---|
| A | Ordinary material | 5.8 gm. (19%) | 18.3 gm. (61%) | 24.1 gm. (80%) |
| B | Coarser " | 7.2 " (24%) | 11.0 " (37%) | 18.2 " (61%) |
| C | Ordinary " | 6.7 " (22%) | 14.0 " (47%) | 20.7 " (69%) |
| D | Coarser " | 6.1 " (20%) | 13.5 " (45%) | 19.6 " (65%) |

* *Phenothiazone expressed as phenothiazine.*

It will be noted that sheep B, which had received the coarser material, excreted a smaller proportion of the drug in the urine. On the other hand, the excretion by D was not significantly different from that of the control animals. While Collier *et al.* (6) showed that the ordinary commercial phenothiazine is not absorbed from the gastrointestinal tract to the same extent as the micronized form, it is apparent from the present experiments that increasing the particle size does not necessarily cause a further decrease in amount of the drug absorbed.

*Mode of Administration*

It had been observed by one of us (22) that, when tablets are administered to the sheep, they tend to drop into the reticulum. It was of interest, therefore, to compare the urinary excretion of phenothiazine after administering the tablets in two ways, namely, (*a*) *per os*, and (*b*) *per fistula* directly into the back of the rumen.

In a further experiment sheep C and D, which had permanent rumen fistulae, were dosed with phenothiazine, C *per os*, and D *per fistula*, using the same dosage as before. The results obtained were similar to those given for these animals in Table II. Again no significant difference was found in the amount of phenothiazone excreted in the urine by the two animals.

*Demonstration of Phenothiazine in the Urine*

As pointed out in the introduction the derivatives of phenothiazine in sheep urine have been studied extensively in several laboratories. All workers agree that the oxidized derivatives predominate. However, Lipson (15) isolated from sheep urine a compound, apparently unaltered phenothiazine, which melted at 180° C. We have confirmed this finding, using the following combination of the method of De Eds (9) for the analysis of urine of the rat, rabbit, and human, and that used by Lipson (15) for sheep's urine.

The combined urines from a previous experiment were acidified with hydrochloric acid. A precipitate formed which was taken up in alcohol and reprecipitated by addition of water. The second precipitate was a pinkish amorphous material which, during a microscopic melting point determination, changed to transparent crystals at 137° C. and melted with darkening in color at 183–4° C. In concentrated sulphuric acid the material showed an absorption band at 515–517 mμ (cf. Figs. 3, 4, and 5). The amorphous material was almost insoluble in 0.1 *N* sodium hydroxide and in 7 *N* hydrochloric acid. It was dissolved in alcohol and decolorized with animal charcoal. On concentrating the solution to a small volume, and cooling, colorless platelets formed. After recrystallization from alcohol the platelets were found to melt at 185–6° C. On heating with 7 *N* hydrochloric acid, the material showed a very faint absorption band at 522 mμ, which did not change with time or on addition of ferric chloride.

It was concluded, therefore, that the product isolated was phenothiazine, and that in the sheep, phenothiazine, or some compound which is readily transformed into it by the addition of hydrochloric acid, is excreted in the urine after the administration of this drug.

## Rumen Studies

*Attempts to Demonstrate Phenothiazone in the Rumen*

The first experiment was designed to ascertain the amount of phenothiazone produced in the rumen after dosing with phenothiazine. The two wethers,

C and D, with permanent rumen fistulae, were dosed with 30 gm. phenothiazine in tablet form. Samples of rumen contents for colorimetric analysis were taken by gentle aspiration through the fistula. The results are given in Table III.

TABLE III

PHENOTHIAZINE IN RUMEN CONTENTS

| Sheep | Hours after treatment | Phenothiazine,* gm. per 100 ml. | "Phenothiazone",* gm. per 100 ml. |
|---|---|---|---|
| C | 3½ | 0.110 | 0.0025 |
| | 24½ | 0.023 | 0.0025 |
| | 48 | 0.0043 | 0.0015 |
| | 71 | 0.0011 | n.d. (not detectable) |
| | 95 | n.d. | n.d. |
| D | 3½ | 0.065 | 0.0020 |
| | 24½ | 0.028 | 0.0035 |
| | 48 | 0.0043 | 0.0033 |
| | 71 | 0.0015 | n.d. |
| | 95 | Trace | n.d. |

** The rumen contents were filtered; the figures for phenothiazine are those obtained with an alcoholic extract of the residue, and the figures for phenothiazone are those obtained from a separate filtration where the filtrate was combined with an aqueous extract of the residue.*

It can be seen that the amounts of phenothiazone formed in the rumen were insignificant. In this experiment, as in many others, the rumen contents, on acidification and addition of ferric chloride, showed no absorption bands at 550 mμ, thus indicating the virtual absence of phenothiazone. It is worth noting, however, that phenothiazine, either in the solid form or absorbed on particles of food, was still present 71 hr. after dosing. Since we were concerned primarily with the absorption of the drug, all experiments reported hereinafter deal with the materials in the liquid fraction of the intestinal contents.

*In vitro Experiments*

The failure to find phenothiazone in the rumen prompted a search for other derivatives of phenothiazine. Rumen contents were collected by gentle aspiration through the fistula into a thermos flask which had been preheated to 40° C. An excess of phenothiazine was added and the mixture incubated at 40° C. Samples, removed periodically, were centrifuged, filtered, and the filtrate extracted with chloroform. The latter extracts were examined with a Beckman spectrophotometer. The absorption spectra of two extracts are shown in Fig. 8. It will be noted that, apart from a small maximum at 280 mμ, which is given by normal rumen contents, only the absorption maximum at 254 mμ, characteristic of phenothiazine, is observable. Again, when unextracted samples were acidified no phenothiazone could be detected. The values given in Table III, for phenothiazone, probably indicate the presence,

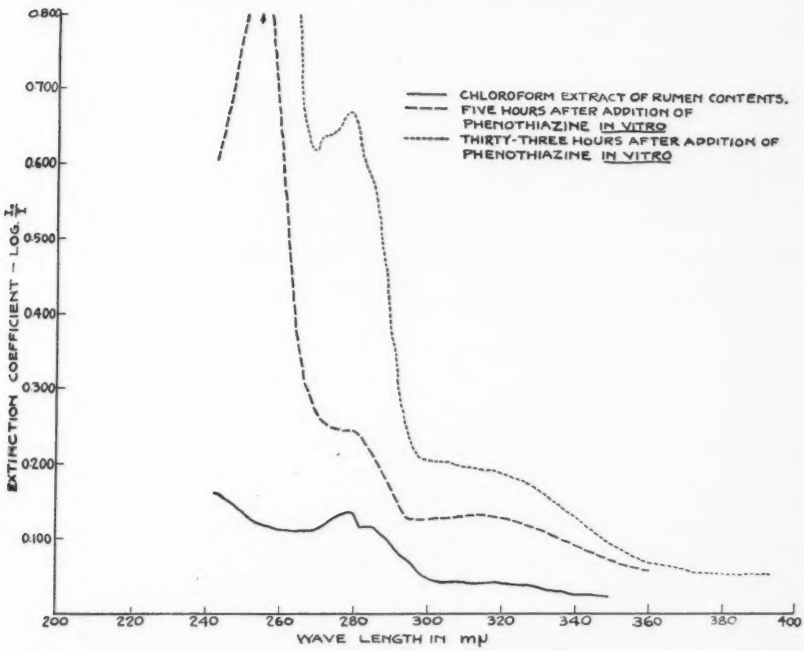


FIG. 8. *Absorption spectra of extracts from rumen contents after addition of phenothiazine in vitro.*

but not the amount, of phenothiazine in solution since the spectra overlap (*vide* Figs. 3 and 4).

*In vivo Experiments*

Similar results were obtained *in vivo*. Twenty grams of micronized phenothiazine, suspended in 2 liters of warm tap water, was introduced *per fistula* into the sheep's rumen. As shown in Fig. 9 the chloroform extracts of the rumen samples reveal only the absorption band of phenothiazine. A chloroform extract, made four and one-half hours after administration, was evaporated to dryness and taken up in 95% ethanol. The amount of phenothiazine found by the bromine method was 0.0015 gm. per 100 ml. of rumen contents. It will be noted in Fig. 9 that at the end of 26 and $\frac{1}{2}$ hr. the phenothiazine band was barely detectable. As solid material was removed from the rumen contents before chloroform extraction, the absorption maxima represent only the phenothiazine in solution. It is interesting to compare the figures given in Table III which indicate that the undissolved phenothiazine may be present in the rumen for a longer period.

These experiments show that the rapid appearance of phenothiazone in the urine cannot be explained by the formation of this compound in the rumen. Until it is known how phenothiazine exerts its anthelmintic action, the possible

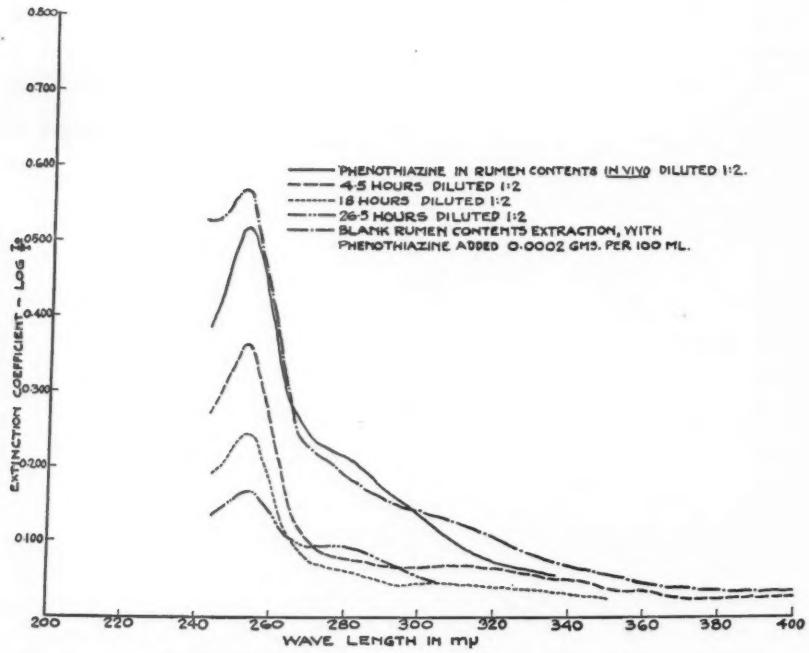


FIG. 9. *Absorption spectra of extracts from rumen contents.*

formation of an "active" compound, in amounts too small to be detected by our methods, must not be overlooked. Nevertheless, in repeated examination of rumen contents of sheep after dosing with phenothiazine, and of rumen contents incubated with phenothiazine, we were unable to obtain any evidence of change in the phenothiazine molecule. However, it is obvious that the drug is more soluble in rumen contents than in water.

## Digestive Tract

In order to investigate the distribution of phenothiazine derivatives in the digestive tract a post-mortem examination was made. A two-year-old ewe weighing 85 lb. was dosed with 32 gm. of phenothiazine in tablet form. Twenty-four hours later the animal was stunned and bled to death. The abdominal cavity was opened and the intestine rolled out and tied in sections to prevent the shifting of the contents.

As the object was to detect the phenothiazine or its derivatives in solution, as opposed to undissolved particles, an attempt was made to collect fluid samples. These were placed in stoppered flasks and kept in the dark until analyzed. All samples were centrifuged as soon as possible and, with the exception of the second duodenal sample, were filtered with the aid of suction before being extracted with chloroform. All extractions were completed

within seven and one-half hours after the death of the animal, and the extracts were stored in stoppered flasks in the dark until examined.

*Examination of Post-mortem Samples*

*Bile*

The bladder bile, on addition of concentrated hydrochloric acid, became bright red in color and showed an absorption band at 557 mμ, characteristic of the phenothiazone free radical, along with a faint band at 500 mμ. We were unable to demonstrate the presence of phenothiazine in the bile. Using the method for the estimation of total phenothiazone in urine (24), the amount found in the bile was 0.036 gm. per 100 ml. This is in agreement with the findings of Collier *et al.* (6).

*Rumen*

A chloroform extract of the filtered rumen contents showed a small absorption maximum at 254 mμ, thus indicating the persistence of a small amount of phenothiazine in the fluid fraction of the rumen contents. Another filtered sample which had been acidified before extraction with chloroform, showed no maximum at 254 mμ. Free phenothiazine is not efficiently extracted from acid solution, but conjugates, if present, may be hydrolyzed under these conditions. The acidified solution, referred to above, showed no band indicative of the free radical of phenothiazone.

*Abomasum*

The extract of the abomasal contents showed a small peak at 254 mμ, but on acidification and further extraction no maximum was observed at the above wave length. The acidified solution developed no red color nor were absorption bands detectable with the Hartridge spectroscope.

*Duodenum*

The chloroform extract of the duodenal contents proximal to the bile duct showed no absorption band at 254 mμ nor could any phenothiazone be detected. The contents distal to the bile duct also were examined but, because of the difficulty in filtering this sample, the fluid was centrifuged and extracted without filtration. The ultraviolet absorption spectrum of the extract indicated the presence of a large amount of phenothiazine (or leucophenothiazone) in the material. On acidification, a considerably larger amount of material was extractable. This was indicated by a more intense relative absorption in the chloroform extract at 254 mμ. The acid solution, on the other hand, showed an absorption band at 555 mμ, indicative of the free radical of phenothiazone. While we obtained no evidence of phenothiazine in the bile, the possibility of its presence must not be overlooked. It is to be expected that the bile salts would increase the amount of phenothiazine in solution or suspension. It is noteworthy, however, that phenothiazone (or leucophenothiazone) was not detected proximal to the bile duct.

*Jejunum*

The absorption band of the free radical of phenothiazone was detected in the acidified solution of the jejunal contents. The ultraviolet absorption spectra of the chloroform extracts were similar to those of the duodenal contents.

*Ileum*

The acidified solution of the filtrate from the ileum contents showed a very faint band at 554 m$\mu$, indicating the presence of phenothiazone. The ultraviolet absorption of the chloroform extracts, both before and after acidification, showed maxima at 254 m$\mu$, but in order to render the solutions readable in the spectrophotometer, less dilution was required than for the jejuna extracts.

*Caecum and Colon*

The contents of these two organs were too extensively dehydrated to permit the use of the usual extraction procedure. After the material had been permitted to stand in stoppered flasks for two hours, an excess of water was added, and the flasks were shaken and allowed to stand for an additional two hours. The samples were then centrifuged, filtered, and extracted with chloroform. The ultraviolet absorption spectra of the extracts made before acidification of the sample, showed distinct maxima at 254 m$\mu$, and those made after acidification showed a faint maximum at the same wave length. The extract of the caecal contents, made before acidification, showed faint maxima at 272 and 305 m$\mu$, which possibly indicate traces of the sulphoxide. In the acidified samples no red color developed. Unfortunately, except in the case of the rumen contents, no data are available concerning the background absorption.

The findings of the post-mortem examination are summarized in Table IV.

TABLE IV

RESULTS OF POST-MORTEM EXAMINATION

| Organ | Phenothiazine | Leucophenothiazone | Acid-hydrolyzable conjugate | Phenothiazone (after acidification) |
|---|---|---|---|---|
| Bladder bile | − | ? | ++++ | ++++ |
| Rumen | ++ | − | − | − |
| Abomasum | + | − | − | − |
| Duodenum | | | | |
| Prox. to bile duct | − | − | − | − |
| Dist. to bile duct | +++++ | ? | +++++ | +++++ |
| Jejunum | + | ? | +++ | +++ |
| Ileum | + | ? | + | + |
| Caecum and colon | ++ | ? | − | − |

## Discussion

The extent to which phenothiazine is oxidized in the rumen of the sheep appears to be variable. Although previous workers have reported the conversion of the drug to phenothiazone (6) and to diphenylamine sulphoxide (4) in the rumen, the results presented in this paper indicate that oxidation does not occur in this organ. It is possible that the fate of the ingested drug is influenced by the diet, either directly or indirectly, since it has been shown that the population of microorganisms in the rumen can be altered by this factor (25). The animals in our study were fed oats and hay. It is highly desirable that the influence of the diet be studied in relation to the oxidation of phenothiazine in the alimentary tract.

While the analyses of the contents of the digestive tract were mainly qualitative, the findings provide the basis for a hypothesis concerning the behavior of phenothiazine in the sheep.

The rapid appearance of derivatives in the urine (6) indicates rapid absorption presumably high in the digestive tract. The fact that no phenothiazone was found proximal to the bile duct suggests that oxidation of the drug takes place after it is absorbed. Subsequent excretion in the bile explains the presence of the oxidized product in the intestinal tract distal to the bile duct. The gradual diminution in the amounts of phenothiazone throughout the small intestine from the bile duct to the caecum, where none was found, probably indicates reabsorption of the oxidized form from the intestine. Concurrently, a large proportion of the oxidized and conjugated products in the bloodstream is excreted by the kidney.

We have carried out only one set of analyses on the entire digestive tract. This experiment, taken in conjunction with the analyses of the rumen contents, indicates that either the oxidized drug is absorbed distal to the bile duct or that the drug is absorbed unoxidized from the rumen. The latter seems the more likely possibility and is supported by the demonstration of the unaltered drug in the acidified urine. Further support for this hypothesis will be presented in a subsequent paper.*

No direct information has been gained with regard to the mode of action of phenothiazine. In our opinion the anthelmintic activity is attributable to the unabsorbed, but not necessarily unaltered, fraction of the drug. Formation of the anthelmintic principle in the tissues of the host and entrance into the alimentary tract by way of the bile duct are unlikely, since even small doses of phenothiazine are extremely effective against the common stomach worm, *Haemonchus contortus*, of the sheep. On the other hand, *Strongylöides papillosus* and *Trichocephalus ovis*, which live in close contact with the tissues of the host, are not affected. If this view be correct, then the anthelmintic activity is not dependent upon absorption of the drug from the intestine.

* *Harpur, R. P., Denstedt, O. F., and Swales, W. E. Can. J. Research, D, 28 : 162-172. 1950.*

Thus, the absorbed drug, which is excreted largely in the urine, is diverted from the site of anthelmintic action. The present work confirms that this loss amounts to more than 40% (23).

It was previously believed that absorption of the drug is favored by oxidation in the rumen. If this were so, the prevention of oxidation would reduce the absorption. However, our findings suggest that phenothiazine itself is absorbed from the rumen and that oxidation in this organ is not essential for absorption.

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# ABSORPTION OF PHENOTHIAZINE FROM THE RUMEN OF SHEEP, AND ITS ESTIMATION IN BLOOD SERUM[1,2,3]

By R. P. Harpur,[4] O. F. Denstedt,[5] and W. E. Swales[6]

## Abstract

Under the conditions described, phenothiazine is shown to be soluble in human and sheep serum. Ultraviolet examination of serum containing phenothiazine indicates that the compound is unchanged by solution in serum. After preliminary experiments with ultraviolet analysis the visual spectra of the free radicals were found to be more suitable for the analysis of sheep serum. A method is described, which makes use of free-radical absorption spectra, for the detection and approximate estimation of phenothiazine in serum when it is present with phenothiazone. The method also allows for the detection of diphenylamine *o*-sulphoxide. Experiments with lambs which had been dosed with phenothiazine showed that while the peripheral circulation contained leucophenothiazone, the blood draining the rumen contained phenothiazine also.

## Introduction

Although it was previously believed (20) that the anthelmintic drug, phenothiazine, is converted to the more soluble derivative, phenothiazone, in the rumen of the sheep, this oxidation does not normally occur in that organ (3, 12). The rapid appearance of phenothiazone in the urine after the oral administration of phenothiazine (20), however, suggests absorption and possibly oxidation, high in the alimentary tract. Clare (3) has reported that some of the drug is oxidized to diphenylamine *o*-sulphoxide in the rumen, but the present authors have not observed this change (12).

It has been pointed out (12) that a study of the fate of phenothiazine in the sheep may yield information of economic importance and that the pharmacology of the drug in other animals requires elucidation.

Since some of the orally administered phenothiazine may be recovered from sheep urine, after acidification (16, 12), it appears possible that phenothiazine itself may be absorbed from the alimentary tract and be oxidized elsewhere in the body. Phenothiazine has been demonstrated in comparatively large amounts in human and pig urine (5), thus suggesting strongly that in these species absorption occurs at the phenothiazine level of oxidation. It appears evident that even in the sheep some phenothiazine may pass into the blood stream and be excreted unchanged in the urine. Up to the present time no method has been reported for the estimation of phenothiazine in blood.

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*Contribution from the Institute of Parasitology, Macdonald College, P.Q., and the Department of Biochemistry, McGill University, Montreal, P.Q., with financial assistance of the National Research Council of Canada.*

[2] *From the M.Sc. Thesis of R. P. Harpur, McGill University, 1947.*

[3] *Presented at the Annual Conference of the Chemical Institute of Canada, Montreal, June, 1948.*

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The present paper describes an investigation of two possible methods for estimating phenothiazine in serum, and the use of one for demonstrating the presence of phenothiazine in the blood draining the rumen of the sheep. As will be shown, one of the difficulties encountered in detecting and estimating phenothiazine in sheep serum arises from the relatively large amount of phenothiazone also present.

## Preliminary Studies

*Methods and Materials*

In addition to those described below, the methods and materials were the same as described in the previous paper (12).

*Solution of Phenothiazine in Serum*

Recrystallized phenothiazine does not dissolve readily in serum, but solutions in this medium were obtained by the following procedures:

To 0.25 ml. of 95% ethanol, containing 0.8 gm. of phenothiazine per 100 ml., was added 1.75 ml. of physiological saline, which precipitated the phenothiazine in a very finely divided form. Eight ml. of pooled human serum (from the blood bank) was then added, mixed, and warmed to 37° C. In a short time the phenothiazine dissolved to give a clear solution.

Qualitative tests for phenothiazine were then made in the following manner:—

One ml. of the serum solution of phenothiazine was added, drop by drop with swirling, to 10 ml. of ethanol–ether (3 : 1). Even after boiling the solution no red color appeared, which would have been observable if oxidation to phenothiazone had occurred. The coagulated protein was removed from the heat-treated solution by filtration, and the filtrate evaporated to dryness on a steam bath in a current of air. There remained a white residue and a small amount of oily material. On the addition of concentrated sulphuric acid, a brown-red solution was obtained, the spectrum of which showed the absorption bands of the free radical of phenothiazine (518 mμ and a faint band of 497 mμ). An extract from a control serum, similarly treated, also gave a brown-red solution in concentrated sulphuric acid but the spectrum showed no absorption bands.

*Attempts to Extract Phenothiazine from Serum*

After having extracted the serum solution of phenothiazine with ethanol–ether as described above, the extract was evaporated and the residue was taken up in 95% ethanol. The amount of phenothiazine was estimated by measuring the red color produced by treatment with bromine. Only 50% of the drug was recovered by this procedure; serum without phenothiazine, treated similarly, was found to be free from interfering colors.

An attempt to facilitate extraction by precipitating the protein in a more finely divided form and by repeated washing instead of warm extraction did not increase the recovery.

On adding phenothiazine to an ethanol–ether extract of serum, and evaporating the solvent, it was found that some of the drug was lost during the evaporation although no apparent oxidation occurred. However, when a solution of phenothiazine in the pure solvent was evaporated on the steam bath in a current of air a purple residue resulted, thus indicating that the serum extract contained some substance which protected the drug against oxidation. This is a significant observation because, when leucophenothiazone was dissolved in the pooled human serum, and allowed to stand for a short time, the red color of the oxidized form appeared.

Later it was discovered that when a solution of phenothiazine in 95% ethanol is evaporated *in vacuo* without heating, no loss occurs provided the evaporation is stopped before the residue becomes completely dry. With this method of evaporation the recovery of phenothiazine from serum was increased to 65%. However, when phenothiazone was used, repeated extraction of the protein precipitate failed to remove completely the red color. Washing with dioxane and chloroform also proved unsuccessful.

From these preliminary experiments it is apparent that the methods for estimating phenothiazine in faeces (4) and in rumen contents (12), which are based upon prior extraction of the drug, are difficult to apply to blood. Further investigation, therefore, was limited to direct spectrophotometric studies of serum containing phenothiazine and its derivatives.

## Methods

The primary objective in this study was to distinguish and measure phenothiazine, phenothiazone, and diphenylamine *o*-sulphoxide in blood serum.* These compounds and their free radicals have distinctive absorption spectra in the ultraviolet and the visual regions, respectively (12; 11, 17, 18). While the spectra of the free radicals proved more suitable for the detection and estimation of phenothiazine derivatives in sheep serum, methods based upon the ultraviolet absorption spectra may be of value in the analysis of the sera of other animals. For this reason the results of ultraviolet analysis will be discussed briefly.

### *Ultraviolet Analysis*

This method is complicated by the overlapping of the absorption bands of the tyrosine and tryptophan in the serum proteins with those of the phenothiazine derivatives. The conditions governing the ultraviolet absorption spectra of proteins have been studied by Holiday *et al.* (13, 14), Goodwin and Morton (10), and by Lerner and Barnum (15). Creech and Jones (6, 7, 8, 9) have applied ultraviolet spectroscopy to the measurement of protein conjugates.

The ultraviolet absorption spectra of phenothiazine derivatives have already been described (12). One example will suffice to illustrate the applicability of the method and the interference by the serum proteins.

* *We desire to acknowledge the helpful advice of the late Dr. L. Michaelis of The Rockefeller Institute for Medical Research, of New York, on this problem.*

A sample of pooled human serum was divided into two portions. Phenothiazine was dissolved in the first portion by the procedure described above, while the second portion, which served as a control, was treated similarly but with the omission of the drug. After suitable dilution of the sera with water the ultraviolet absorption spectra were examined with a Beckman spectrophotometer, Model B. It was found that when the absorption coefficients for the control serum were subtracted from those of the serum containing phenothiazine, the resulting values represent the spectrum of the added phenothiazine. By the same procedure the absorption spectrum of diphenylamine *o*-sulphoxide was obtained. The results are illustrated in Fig. 1. It

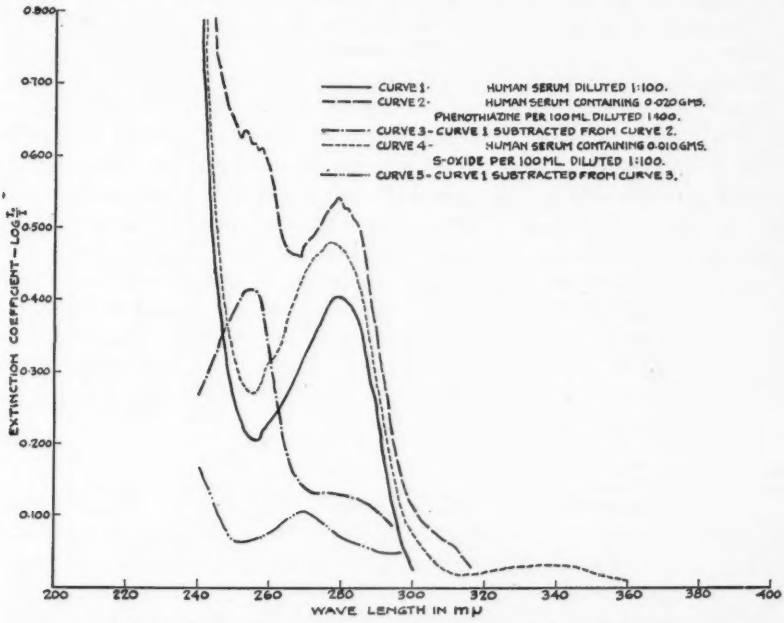


FIG. 1. *Effect of phenothiazine and diphenylamine o-sulphoxide on the absorption spectrum of human blood serum.*

will be noted in this figure that when phenothiazine, or the sulphoxide, is dissolved in serum the ultraviolet absorption bands remain unaltered. This affords further support of our contention that phenothiazine may be transported unchanged in the blood stream. However, the ultraviolet was not investigated further because the concentration of phenothiazine in the sera of sheep, after dosing with phenothiazine, was found to be too small to be measurable in the presence of the serum proteins.

*Analyses by Visual Spectra*

For the estimation of phenothiazone in serum, Collier *et al.* (4, 21) utilized the light absorption of its free radical in acid solution. This method offers

the advantage that by measuring the color change from the oxidized to the reduced state, the interference of other chromogenic substances in the serum is minimized. This method, however, has not been adapted previously to the estimation of phenothiazine.

In acid solution phenothiazine may be distinguished from phenothiazone by the difference in the absorption spectra but diphenylamine *o*-sulphoxide possesses a separate spectrum only in concentrated acid (12). The sulphoxide had to be considered since Clare (3) has reported its presence in the portal blood of sheep after dosing with phenothiazine. Because of the similarity of the spectra of phenothiazine and the sulphoxide it was anticipated that a separate method for the detection of the sulphoxide would be necessary. It was found, however, that when acidified serum, containing the sulphoxide, was reduced, the orange solution became colorless, as do acid solutions of phenothiazine and phenothiazone. Unlike the latter two, however, the colorless solution became purple on standing, and the color was unaltered by further addition of stannous chloride. This property was used as a test for the sulphoxide, since only in cases where the latter had been added to the serum did the purple color appear. On the other hand, when phenothiazine is allowed to stand in acidified serum for some time before being reduced with stannous chloride, a purple color appears after reduction. Furthermore, when ferric chloride is added to phenothiazine in acid solution and allowed to stand, the extinction coefficients at the frequencies used were observed to increase with time. The extinction coefficients of acid solutions of phenothiazone, on the contrary, decrease with time. Thus we found that the following procedure gave the most reproducible results.

*Procedure*

A sample of serum (0.1–2.0 ml.) was placed in a colorimeter tube and diluted to 2 ml. with water. Four ml. of concentrated hydrochloric acid was added and one drop of 1% ferric chloride in 8 *N* hydrochloric acid. The extinction coefficient was determined at once with an Evelyn photoelectric colorimeter, first with a 520 m$\mu$ filter ($R_1520$) and then with a 565 m$\mu$ filter ($R_1565$). Immediately afterwards, a crystal of stannous chloride was added and the tube was shaken. The solution was allowed to stand for one hour to permit the detection of the sulphoxide by the purple color developed in its presence. Another crystal of stannous chloride was then added and the extinction coefficient again determined at 520 m$\mu$ and 565 m$\mu$ ($R_2520$ and $R_2565$).

The extinction coefficients of the solutions, after reduction, were subtracted from those obtained after the addition of ferric chloride. Calculations were made from the resulting values ($R_1$–$R_2$)520 m$\mu$ and ($R_1$–$R_2$)565 m$\mu$. If only the sulphoxide is present the value, ($R_1$–$R_2$)565 m$\mu$, is negative. As a purple color was not encountered with any of the serum specimens tested there was no necessity for developing a method for estimating the sulphoxide.

*Determination of Extinction Coefficients*

By the procedure just described it was possible to obtain fairly reproducible values for the molecular extinction coefficients of phenothiazine and phenothiazone dissolved in serum. Serum from haemolyzed blood was found to give unsatisfactory results. Normal human and sheep sera were found to give negligible values for $(R_1-R_2)520$ m$\mu$ and $(R_1-R_2)565$ m$\mu$. Average values for the molecular extinction coefficients ($\epsilon$) of phenothiazine and phenothiazone in serum are given in Table I.

TABLE I

MOLECULAR EXTINCTION COEFFICIENTS ($\epsilon$) FOR DIFFERENT MEDIA BY THE METHOD DESCRIBED

| Medium | Phenothiazine | | Phenothiazone | |
|---|---|---|---|---|
| | $\epsilon_{520m\mu}$ | $\epsilon_{565m\mu}$ | $\epsilon_{520m\mu}$ | $\epsilon_{565m\mu}$ |
| Pooled human serum | 4460 | 280 | 3060 | 5030 |
| Fresh human serum* | 2715 | 0 | 4050 | 5320 |
| Fresh sheep serum | 4720 | 260 | 6570 | 5770 |

* *The addition of nembutal did not affect the values.*

It will be noted that the values differed with the serum used. Since these values were obtained by subtracting the extinction of the reduced form from that of the oxidized form, it appears that the oxidation and reduction processes are catalyzed to a different degree in different sera.

*Calculations*

It will be noted in Table I that the value of $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ for phenothiazone is close to unity. On the other hand, for phenothiazine the ratio is greater than one. Therefore, in mixtures of phenothiazine and phenothiazone the presence of the former is indicated when the above ratio is greater than one.

Furthermore, the quantitative estimation of phenothiazine and phenothiazone in mixtures can be calculated from the readings at the two frequencies.

The method of calculation for binary mixtures was first described by Vierordt and has been discussed by Twyman and Allsopp (22). Holiday (13) and Goodwin and Morton (10) also have used it and have discussed considerations relative to the choice of frequencies.

Unfortunately, the procedure which has been outlined for serum is of low accuracy; nevertheless it does afford a rough estimate of the concentration of phenothiazine and phenothiazone. This was demonstrated by using known mixtures of these compounds.

## Results

A 55 lb. ram lamb was dosed with 24 gm. phenothiazine in tablet form (19), and two hours later the animal was anesthetized with nembutal. The lamb

was laid on its left side and an incision made in the right side, parallel to the last rib for a length of about nine inches. The incision was extended caudally for a further five inches. The rumen and abomasal veins were exposed by blunt dissection.* Blood samples were collected from the following sites: jugular vein, carotid artery, rumen vein, and abomasal vein. All serum specimens were treated according to the procedure described above. The ratios obtained from the extinction coefficients and the calculated concentration of phenothiazine and phenothiazone are shown in Table II. Unfor-

TABLE II

ANALYSIS OF BLOOD SERUM AFTER ADMINISTRATION OF PHENOTHIAZINE (EXPERIMENT 1)

| Blood sample | $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ | Phenothiazine, gm. per 100 ml. | Leucophenothiazone, gm. per 100 ml. |
|---|---|---|---|
| Carotid | 1.06 | 0 | 0.006 |
| " | 1.05 | 0 | 0.007 |
| Rumen | 1.66 | 0.002 | 0.004 |
| " | 1.66 | 0.002 | 0.004 |
| Abomasum | 1.21 | 0.0005 | 0.004 |
| " | 1.24 | 0.0006 | 0.004 |
| " | 1.27 | 0.0005 | 0.003 |
| " | 1.26 | 0.0004 | 0.003 |

tunately, all the samples taken from the jugular vein were somewhat haemolyzed, and as haemolyzed blood does not give consistent readings with our method, the values have been omitted. It is noteworthy, however, that the values $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ obtained with the jugular samples varied within the range 1.15–1.23.

After the blood samples had been collected, the animal was sacrificed. The acidified rumen contents and acidified abomasal contents showed the absorption band of the free radical of phenothiazine at 518 m$\mu$, but no band was visible in the 550 m$\mu$ region.

Thus, the experiment confirmed that no phenothiazone was present in the rumen or abomasum. While the calculated concentrations in Table II are not of high accuracy it is evident from the values for $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ that the ratio of phenothiazine (or some compound at the same oxidation level) to leucophenothiazone in the blood from the rumen was considerably higher than in the peripheral blood.

The experiment was repeated maintaining the same conditions as to weight of lamb, dose, time, etc. The results obtained are shown in Table III.

** We wish to thank Prof. B. P. Babkin, and Dr. Dorothy Karp, Department of Physiology, McGill University, for performing the operation.*

TABLE III

ANALYSIS OF BLOOD SERUM AFTER ADMINISTRATION OF PHENOTHIAZINE (EXPERIMENT 2)

| Blood sample | $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ | Phenothiazine, gm. per 100 ml. | Leucophenothiazone, gm. per 100 ml. |
|---|---|---|---|
| Jugular—before nembutal | 0.99 | 0 | 0.006 |
| | 1.00 | 0 | 0.006 |
| | 1.02 | 0 | 0.006 |
| Jugular—after nembutal | 1.01 | 0 | 0.004 |
| | 1.08 | 0 | 0.004 |
| | 1.03 | 0 | 0.005 |
| | 1.19 | *ca.* 0.0004 | 0.005 |
| | 1.16 | *ca.* 0.0003 | 0.005 |
| | 1.15 | *ca.* 0.0002 | 0.004 |
| Carotid | 1.17 | *ca.* 0.0002 | 0.003 |
| | 1.11 | 0 | 0.003 |
| | 1.11 | 0 | 0.003 |
| Rumen | 1.62 | 0.003 | 0.004 |
| | 1.66 | 0.003 | 0.004 |
| | 1.61 | 0.003 | 0.004 |
| | 1.61 | 0.003 | 0.004 |
| | 1.58 | 0.002 | 0.004 |
| Abomasum | 1.22 | *ca.* 0.0003 | 0.002 |
| | 1.11 | 0 | 0.004 |

It will be observed that the results are consistent with those in Table II except that there is no significant difference between the ratios for the abomasal vein and the peripheral blood.

To check further the validity and accuracy of these results a large quantity of blood was collected from the carotid artery of the second animal at the conclusion of the experiment. To portions of the serum were added various amounts of phenothiazine until values for $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ were obtained which were similar to those given by the blood from the rumen. The results obtained with the carotid serum are shown in Table IV.

It can be seen that the addition of 0.0016 gm. of phenothiazine per 100 ml. of serum increases the ratio, $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$, to a value comparable with that obtained with the rumen blood. Furthermore, a comparison of Tables III and IV shows that the calculated concentration of phenothiazine in the rumen sample agrees favorably with the amount of phenothiazine that was added to the carotid serum in order to increase the value of $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ to 1.5. If diphenylamine *o*-sulphoxide is present (in a concentration equivalent to that calculated for $R_2$ phenothiazine) a purple color is produced prior to taking the second ($R^2$) reading. None of the serum samples in these experiments showed this color.

TABLE IV

ESTIMATION OF PHENOTHIAZINE ADDED TO CAROTID BLOOD SERUM*

| Phenothiazine added, gm. per 100 ml. | $\frac{\epsilon_{520m\mu}}{\epsilon_{565m\mu}}$ | Calculated | |
|---|---|---|---|
| | | Phenothiazine, gm. per 100 ml. | Leucophenothiazone, gm. per 100 ml. |
| 0 | 1.15 | 0.0001 | 0.002 |
| 0 | 1.13 | 0 | 0.002 |
| 0.004 | 2.19 | 0.003 | 0.002 |
| 0.004 | 2.23 | 0.004 | 0.002 |
| 0.0016 | 1.48 | 0.002 | 0.004 |
| 0.0016 | 1.54 | 0.002 | 0.004 |
| 0.0008 | 1.28 | 0.0007 | 0.003 |
| 0.0008 | 1.28 | 0.0007 | 0.003 |

* *The serum was diluted 1.25 times with 95% ethanol and saline before treatment with acid.*

This method, though indirect, demonstrated that phenothiazine was present in the blood from the rumen and was virtually absent from the other samples examined. It appears, therefore, that phenothiazine, or a substance which behaves like it when treated with acid, is absorbed by the rumen and may be oxidized elsewhere in the body.

## Discussion

In this and the previous paper (12), methods have been described for detecting, measuring, and distinguishing phenothiazine, diphenylamine *o*-sulphoxide, and phenothiazone. These methods have been used to study the absorption of the drug from the rumen of the sheep. The studies have shown that phenothiazine normally is not oxidized to phenothiazone in the rumen, a finding which is in agreement with the observations of Clare (3). However, contrary to Clare, who found the sulphoxide in the rumen, we found only phenothiazine.

Conditions in the alimentary tract are predominantly anaerobic (2). However, there is the possibility that the phenothiazine in the rumen contents may undergo oxidation if it comes in contact with air in the presence of the slightly alkaline saliva, during regurgitation. Diphenylamine *o*-sulphoxide is known to be formed from phenothiazine by oxidation in alkaline solution (1). Clare (3) detected the sulphoxide in the portal blood also after dosing with phenothiazine. Comparing our observations with those of Clare and of Collier *et al.* (5) it seems possible that the extent of oxidation in the rumen of the sheep is variable.

With regard to the oxidation level of the drug, it is interesting to note that the urine of a sheep after dosing with phenothiazine, although normal in color when voided, rapidly turns a deep red color (4). On the other hand, the serum shows no tendency to oxidize in this manner when exposed to the air (4).

As mentioned earlier, when leucophenothiazone was placed in pooled human serum it gradually turned red. Some interesting information might be gained by a further study of these phenomena.

From our results it appears that in the sheep phenothiazine behaves in the following manner: The first absorption of the drug takes place from the rumen; it is doubtful whether any absorption occurs from the abomasum. (Other sites were not investigated.) Some phenothiazine (or very closely related compound) is excreted in the urine. The drug is oxidized either after absorption from the alimentary tract or in the tract distal to the bile duct. The product of oxidation is leucophenothiazine, some of which undergoes conjugation. These products are excreted into the urine and in the bile.

It is hoped that the methods outlined in this paper may provide a basis for further studies on the fate of phenothiazine in the sheep and also in other animals.

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# THE MORPHOLOGY AND DEVELOPMENT OF THE SHEEP NEMATODE, *STRONGYLOIDES PAPILLOSUS* (WEDL, 1856)[1]

By M. A. Basir[2]

## Abstract

In studying the morphology and development of *Strongyloides papillosus* it has been shown that four molts occur during the development of the free-living sexual adults, two molts in the formation of the infective larvae, and two molts in the development of the parasitic adults from the infective larvae. The last two occur in the body of the host, one in the lungs and the other in the intestine. The time required by the eggs to hatch and the larvae to develop to either the infective larvae or the free-living adults varies with temperature; at 27° C. it is 6 hr. and 28 hr., respectively. The so-called "spears" in the oesophagus of the free-living adults were found to be the cuticularized tubular endings of the rays of the oesophageal lumen. The head in the free-living adults bears two well developed lips and four papillae, while in parasitic adults it has four lips and four papillae. The tail of the free-living male bears two pairs of preanal and two pairs of postanal papillae. No males were found in the parasitic generation, while parasitic females were found only in the intestine of experimental animals.

The genus *Strongyloides* offers problems of an intricate and complex nature in nematode biology. It is one of the few worms which combines a parasitic as well as a free-living adult generation in its life cycle, and, owing to its ability to infect laboratory animals and its very common occurrence in domestic and wild animals as well as in man, it has attracted considerable attention from workers in parasitology and biology.

Wedl (12) described a number of hairlike female worms from the intestine of sheep and called them *Trichosoma papillosum*. Later it was found that these belonged to the genus *Strongyloides* and were transferred to this genus as *S. papillosus* by Ransom (10). This is, therefore, the oldest known species of the genus, the human species being discovered much later. However, it was the discovery of the human form that stimulated extensive research into the life history and associated problems of this member of this genus.

The parasitic females of *Strongyloides* live more or less embedded in the mucosa of the small intestine where they lay eggs. The eggs either hatch in the intestine as in *S. stercoralis* or are passed out with the faeces as in *S. papillosus* and related species and hatch outside the body of the host. The larvae may develop along two different directions. They may either metamorphose directly into infective larvae or they may grow into a free-living generation of males and females which in turn produces a progeny of infective larvae. In the former case the development is known as "direct" or "homogonic" and in the latter case "indirect" or "heterogonic".

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*Contribution from the Institute of Parasitology, Macdonald College (McGill University), Macdonald College P.O., Que. This investigation was briefly reported at the Pacific Science Congress, held in New Zealand Feb. 3, 1949.*

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In spite of extensive studies by various workers on various species much difference of opinion still exists on several points of their life history and biology. In particular, little attention has been paid to the development of *S. papillosus* while there is no detailed account of its morphology in the literature.

## Materials and Methods

Throughout the investigation, faeces from a lamb infected with *S. papillosus* were used for the study of the free-living generation of this species. It was found that if faeces were collected after deposition on the floor they were readily contaminated with free-living nematodes, and owing to their similarity with the free-living sexual generation of *Strongyloides*, this caused considerable confusion. To avoid this, faeces were taken directly from the rectum of the sheep thereby eliminating all possibilities of external contamination. To procure infective larvae for infecting experimental animals, routine faecal cultures were made by mixing faeces with an equal amount of sand or charcoal and keeping them in Petri dishes. It was found to be essential that the layer of faeces in the dish be very thin and the faeces just moist; even slight excess of water proved to be detrimental to a healthy growth of the free-living generation. The lid of the Petri dish was lined with a filter paper, kept moist in order to maintain the required humidity. Most of the infective larvae produced migrated upwards and lay in a quiescent state on the filter paper, from which they were easily collected. If the whole population was required, the Baermann technique was used to collect the individuals from the faecal mass.

As the time of molting varies considerably at different temperatures, cultures for such studies were incubated at a constant temperature of 27° C. unless otherwise stated. At 27° C. most of the eggs hatch in about six hours, and so the age of the larvae was counted from six hours after the collection of the faeces.

The life cycle of the free-living generation of this species took about 28 hr. to complete at 27° C. Therefore, it was not possible to study the complete life cycle continuously. Faeces were collected and cultures were made at 8 a.m.; most of the eggs hatched at 2 p.m. From 2 p.m. to 11 p.m., i.e., from the age of one hour to the age of nine hours, larvae were studied hourly. The next morning the study was started at 8.am. and continued up to 10 p.m., i.e. larvae were studied from the age of 19 hr. to the age of 31 hr. On the following day faeces were collected and a second series of cultures made at 10 p.m.; most of the eggs in this series of cultures were presumed to have hatched at about 4 a.m. Examination of these larvae was started at 8 a.m. and continued till 11 p.m., i.e. the ages from 6 to 20 hr. were covered. In this way, by taking faeces at different hours, all ages of the larvae were studied.

For the study of the molting within the host, rabbits were used as experimental animals, as it was known from the investigations of Brumpt (2) and Sandground (11) that these animals contract *S. papillosus* infection normally.

As it was found that fixation of worms in alcohol resulted in considerable shrinkage of their bodies and a consequent change in their size, worms were not killed but studied alive, immobilized by heat. This procedure left their bodies stretched and transparent so that all details could be drawn; all measurements were made on such specimens.

## Life History of *Strongyloides papillosus*

*Modes of Development of the Free-living Generation*

Investigations of other workers on *Strongyloides papillosus* have shown that in the free-living generation, this species exhibits a mixed type of development i.e., a part of the progeny of the parasitic females develops directly into infective larvae and a part into free-living unisexual adults. It has also been shown by Brumpt (2) and Sandground (11) that in the free-living unisexual adults an overwhelming majority consists of females while the males are few or even absent. They also found that if this species is transferred to rabbits, the mode of development of the free-living generation undergoes a remarkable change. The development becomes almost totally indirect and the number of males nearly equals the number of females. The present studies confirm these findings.

## Development of the Free-living Adults from the Egg of the Parasitic Female

*Eggs*

The eggs are passed in the faeces of the infected sheep. They are ellipsoidal in shape, thin-shelled, and measure 56 to 64$\mu$ in length by 34 to 42$\mu$ in width (Fig. 4, F). Owing to the continuous wriggling of the larva inside the egg the shape of the eggs varies considerably, with the result that in certain cases they become more elongated than usual.

The time required for hatching is variable and depends primarily on the temperature of the medium rather that the nature of the medium itself. Eggs placed in various media of different pH hatched normally. In water or Lock's solution, they hatch in about four hours at a temperature of 30° C., in about six hours at 25° C., and in about 8 to 10 hr. at 20° C.

*First-stage Larva*

Freshly hatched larvae (Fig. 1, A) vary in length from 235 to 290$\mu$. They are not very slender and may be termed "tadpole" shaped. They have a maximum width of about 16$\mu$ at the base of the oesophagus from where the body gradually tapers towards the anterior and posterior ends. The head end of the larva is broad and is about 8$\mu$ wide in the region of the buccal cavity. Posteriorly the body ends in a pointed tail. The cuticle shows no apparent striations. The buccal cavity is cylindrical, 5$\mu$ long by 1.3$\mu$ in width. The larva shows a separation of cuticle at the anterior end, suggesting that the process of the first molt had already started while the larva was inside

the egg. The oesophagus is 66 to 88$\mu$ long and is divisible into four parts: (1) an anterior vestibule measuring 9$\mu$ in length by 6$\mu$ in width, which is in the form of a subspherical muscular portion and is cut off from the remainder of the oesophagus by a constriction; (2) a club-shaped rhabditiform bulb 33.5$\mu$ long by 11.5$\mu$ wide near its base; (3) a long narrow isthmus 18$\mu$ long by 3.5$\mu$ wide, which merges imperceptibly into (4) a posterior valvular bulb 13.5$\mu$ long by 11.5$\mu$ wide. The intestine is functional and is subcylindrical in shape. The rectum is 6$\mu$ long. The anus lies at a distance of 46.5$\mu$ from the posterior end of the body. The tail gradually narrows behind the anus and ends in a point. The nerve ring is situated in the middle of the isthmus, 54$\mu$ from the anterior end of the body. The genital primordium lies at a distance of 125 to 140$\mu$ from the anterior extremity, slightly posterior to the middle of the body. It is oval in shape and is 14 to 22$\mu$ long. It shows no distinct division into cells in a newly hatched larva.

As those larvae become older, they increase in size gradually but their relative morphology remains the same. The cuticle in the head region separates more and more. In the tail region no progressive separation of the cuticle was seen but at the age of between 7 and 10 hr., the cuticular sheath starts coming out of the tail end (Fig. 1, B). The molting sheath is so fine that it requires very careful observation to see it, and it takes so little time in passing from the body completely, that it has to be seen at the right moment. The larva wriggles and in a few jerks the whole cuticle is thrown off. The major portion of the casting sheath is passed by the tail end, with only a small portion from the head end. The size of the larvae at the time of this molt is between 325$\mu$ to 385$\mu$.

All the newly hatched larvae are similar in morphology and no difference could be made out by which larvae of the direct and indirect generation could be distinguished at the time of hatching. However, after a few hours,

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FIG. 1. *The free-living phase of Strongyloides papillosus.*

*The indirect cycle:*

*A. Rhabditiform larva, just hatched, lateral view.*
*B. Larva in process of the first molt.*
*C. Female larva in process of the second molt.*
*D. Female larva in process of the third molt.*
*E. Female larva in process of the fourth molt.*
*F. Male larva in process of the second molt.*
*G. Male larva in process of the third molt.*
*H. Male larva in process of the fourth molt.*
*I. Adult male, lateral view.*
*J. Male, en face view.*
*K. Male, anterior end, lateral view.*
*L. Male, tail, ventral view.*
*M. Spicules with gubernaculum.*
*N. One spicule.*
*O. Gubernaculum.*

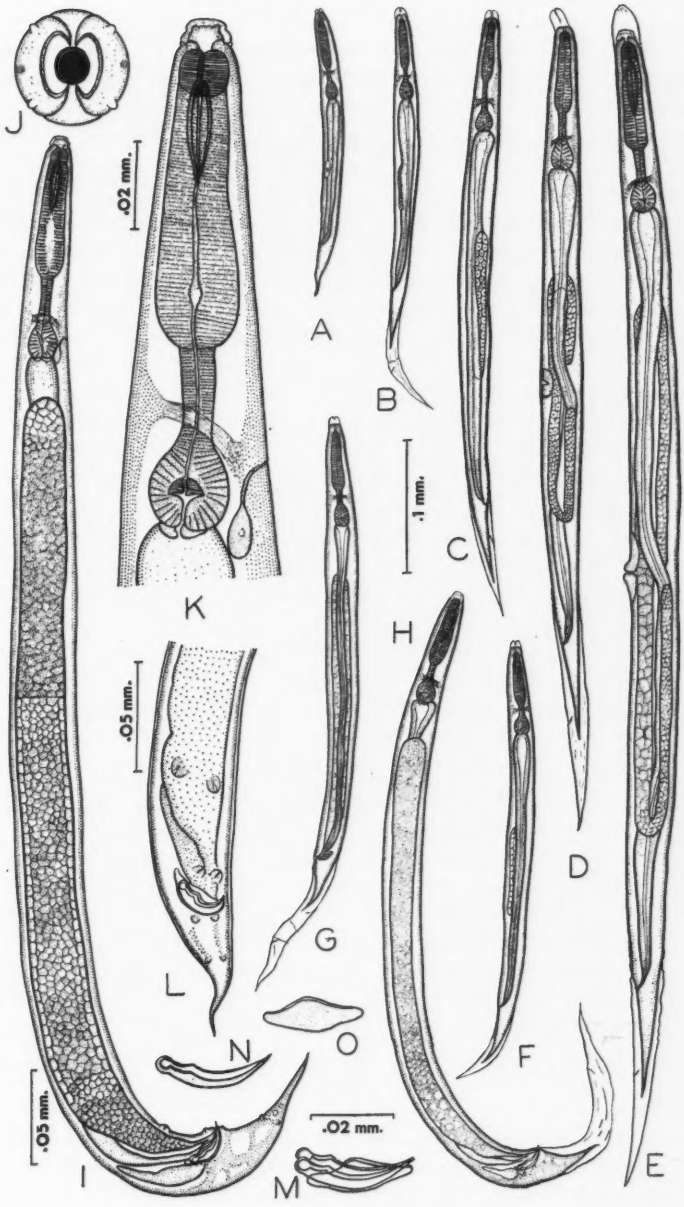



FIG. 1.

by careful observation such distinction can be seen. In the larvae of the sexual generation, the cell of the genital primordium begins to divide, with the result that the latter increases in size and the number of cells forming it gradually increases in number. At the time of the molt the genital primordium measures about 35$\mu$ in length.

The sexes cannot be differentiated at this stage.

*Second-stage Larva*

After the first molt has taken place, the larvae can easily be distinguished from those in which the molt has not taken place, by the absence of any separation of cuticle on the head in the former. However, this state exists only for about an hour or two and separation of cuticle on the head again starts. The larvae progressively grow in size and, during this stage, distinction can easily be made between the larvae of the direct and the indirect generation. The latter are thicker and more robust than the former. In the larvae of the indirect generation the genital primordium increases in length and lies on the ventral side of the body.

The separation of the cuticle on the head progressively increases while on the tail it could not be seen until the molt. Larvae were seen undergoing the molt between the ages of 14 and 16 hr. (Fig. 1, C). The size of the larvae at this stage is between 425 and 475$\mu$ in length by 28$\mu$ in maximum width as compared to a width of 16$\mu$ in the larvae of the direct generation at the same age. A larva 450$\mu$ long at the time of the molt gave the following measurements: oesophagus 85$\mu$ long, anus 60$\mu$ from posterior end; genital primordium 115$\mu$ long. In some larvae, the ends of the genital primordium were already reflexed while in others they were still straight. The nerve ring lies in the region of the isthmus between the base of the first bulb and the beginning of the second one. The buccal cavity is cylindrical as in the first-stage larvae.

As the second-stage larva grows and reaches the stage of casting its cuticle, the sexes can be differentiated to some extent. In the female larvae the elongated genital primordium lies clearly obliquely on the ventral side of the intestine, its anterior half lying on the right side of the intestine and its posterior half on the left side. In the male larvae (Fig. 1, F) its position is not oblique; it lies straight and parallel to the intestine either completely on its ventral side or on its right ventrolateral side. The spicules are not yet apparent and the tail is still as in the female larvae. In size the male larvae are slightly smaller than the female ones.

*Third-stage Larva*

*Female*

After the second molt has taken place, the third-stage larvae can easily be recognized and differentiated from previous stages by their broad and blunt anterior ends and by the size of the genital primordium, both ends of which are now reflexed. The buccal cavity gradually loses its long cylindrical shape and becomes more or less funnel-shaped. Gradually the body wall near

the center of the genital primordium thickens inwards to form a convex padlike structure which presses against the genital primordium. A slit develops in it towards the outside and gradually grows inwards but this is covered externally by a double cuticular layer; this slit represents the growing vulva and is very typical of the fully grown third-stage larvae which are approaching the molt.

Larvae are seen in process of molting at the age of about 21 hr. (Fig. 1, D). The length of the larvae at this stage varies from 525$\mu$ to 560$\mu$. A larva 540$\mu$ long with a maximum width of 32$\mu$ gave the following measurements: oesophagus 110$\mu$ long; anus 75$\mu$ from posterior extremity; vulva 270$\mu$ from anterior end of body; genital primordium occupies a length of 390$\mu$; its anterior half lies on the right side of the intestine and its posterior half on its left side; anterior flexure of ovary 80$\mu$ behind base of oesophagus, posterior flexure of ovary 90$\mu$ in front of anus.

*Male* (Fig. 1, G)

Larvae of this stage can easily be classified into males and females. The male larvae are much smaller in size. The genital primordium in the males is not flexed at its ends and it lies straight, completely on the right side of and parallel to the intestine. There is no padlike thickening with the developing vulvar slit characteristic of this stage in the female. When the larvae are killed, although the tail does not completely adopt the typical curved shape of the adult male, still it has a typical structure, tapering very suddenly behind the cloacal opening. Traces of spicule formation can be observed but these are not yet heavily cuticularized.

*Fourth-stage Larva*

*Female*

After the third molt, the larvae appear like young adults and gradually develop all adult characters. At the age of about 28 hr. larvae were found undergoing the fourth molt (Fig. 1, E). Their length at the time of the molt varies from 775 to 820$\mu$. A larva 820$\mu$ long gave the following measurements: maximum width 40$\mu$; oesophagus 138$\mu$ long, vulva 415$\mu$ from anterior extremity, anus 85$\mu$ from posterior end of body, flexure of the anterior ovary 65$\mu$ behind base of oesophagus, and that of posterior ovary 115$\mu$ anterior to the anus. The ovaries have lengthened and have nearly assumed the adult character. The vulva is completely formed but before the molt it is seen to be covered externally by a thin cuticular layer. The uterus has not yet developed a lumen. The head has already assumed the adult structure.

*Male* (Fig. 1, H)

At the approach of the fourth molt the larvae are nearly fully developed adult males with a full grown testis. The anterior end of the testis extends up to a point 25$\mu$ behind the base of the oesophagus. The spicules and the gubernaculum are fully developed and, on killing the larva, the tail assumes the typical curve as in the adult.

*Adults*

*Female* (Fig. 2, G–J)

Within a few hours after the final molt the worms develop all adult characters, the uterus develops a lumen and eggs are seen in it.

Fully grown females vary in size from 770$\mu$ to 1.11 mm. in length and from 50 to 90$\mu$ in maximum width. However, the measurements in the following description are taken from a female worm measuring 1.01 mm. in length and which had a maximum width of 55$\mu$ near the middle of the body. The body itself is quite plump and is broadest in the region of the vulva. It remains more or less cylindrical in the region of the reproductive organs and tapers perceptibily both anterior to the flexure of the anterior ovary and posterior to that of the posterior ovary.

The cuticle is not apparently striated but under high magnifications very light and minute striae may be noticed.

The head (Fig. 2, B) is distinctly separated from the body by a constriction, and shows an expansion in the form of a crown which is 10$\mu$ high. The width of the body at this constriction is 12$\mu$. The form of the head structures of *Strongyloides* has not yet been clearly described. Kreis (6) writes that the head "probably has six lips, which are not very distinctly developed. The lips of the female in the canine strain are flattened so strongly that they can hardly be seen. No papillae could be observed even under the highest magnification." Alicata (1) is the only worker who has shown the presence of only two lips in the free-living generation. In the present study, an *en face* view (Fig. 2, C) showed that the head has no resemblance to that of a rhabditid. It has two distinct and well developed lateral lips, each of which bears two papillae, one on its dorsolateral aspect and the other on its ventrolateral side. Amphids appear as comparatively large openings on the lateral sides of the lips and can also be seen in a lateral view of the worm. If the worm is examined under a fairly high magnification from the dorsal or ventral aspect, the structure of the lips can be studied but an examination of the worm as is usually done in a lateral view, gives a confusing idea about the form of the lips.

FIG. 2. *The free-living phase of Strongyloides papillosus.*

*The free-living adult female:*

*A. Anterior end, dorsal view.*
*B. Oesophageal region, lateral view.*
*C. En face view.*
*D. T.S. anterior oesophageal region.*
*E. Region of vulva, lateral view.*
*F. Region of vulva, ventral view.*
*G. Young female with a single row of eggs, lateral view.*
*H. Fully mature female with two rows of eggs in the uterus.*
*I. Female, dorsal view, showing the disposition of the intestine.*
*J. Female, postfunctional, lateral view.*

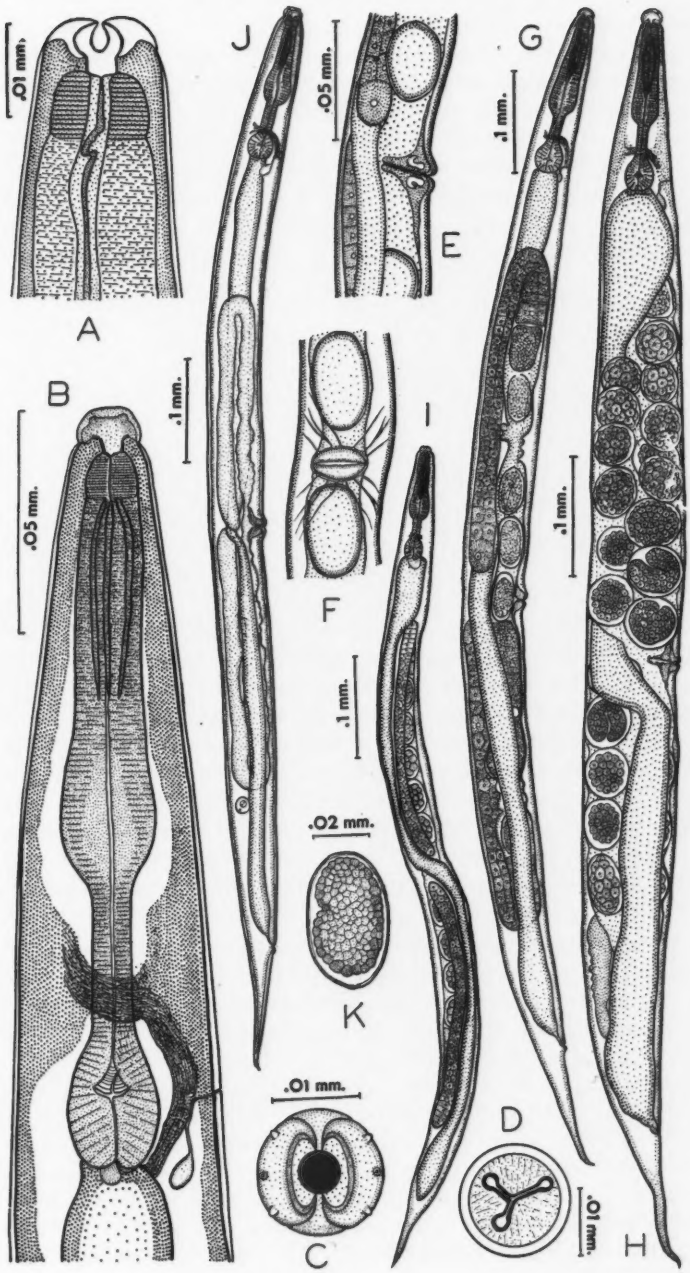



FIG. 2.

The form of the lips and the disposition of the cephalic papillae in this stage are of considerable interest. It is generally thought that among Nematoda, *Strongyloides* represents an evolutionary stage showing transition from a free-living form that has not yet been completely suppressed and still can occasionally be seen. But the study of the head structures indicates that these worms might be highly evolved and be comparatively older parasites, where a free generation has secondarily developed in order to facilitate the multiplication of the infective larvae under favorable conditions of environment. Thus the apparent rhabditiform structure of the worm may merely be a phenomenon of evolutionary convergence due to similar environmental and nutritional conditions in which the true rhabditids live.

The buccal cavity may be divided into two parts: one part which is enclosed by the lips is globular and is about 10$\mu$ deep and the other part is funnel-shaped and is only 3$\mu$ in depth. The funnel-shaped portion probably represents the true stoma and is lined by thick cuticularized walls showing two distinct sets of rhabdions. The buccal cavity in the adult has no resemblance to that of the free-living true rhabditids.

The oesophagus is 140$\mu$ long and is divisible into four main parts. A small anterior bulblike muscular portion, which is 10$\mu$ long by 12$\mu$ broad and which is generally termed the vestibule. The vestibule is followed by a club-shaped rhabditiform bulb which has a length of 78$\mu$ behind the vestibule and has a maximum width of 22$\mu$ near its base. This in turn is followed by a narrow isthmus 22$\mu$ long by 8$\mu$ wide. The latter merges imperceptibly into a more or less pear-shaped posterior valvular bulb which is 25$\mu$ long by 22$\mu$ wide. Kreis (6) reported and figured three well-developed spears in the anterior oesophagus. He even went as far as to suggest possible functions for these. Such spearlike structures are to be seen in these worms but a careful examination reveals that they are not spears but the tubular ends of the rays of the oesophageal lumen. In true rhabditids the ends of the rays of the oesophageal lumen terminate in incomplete longitudinal tubes and such tubes are present in these worms also (Fig. 2, B–D). These appear as spearlike cuticular rods when seen in a lateral view.

The oesophagus communicates with the intestine through a pair of highly developed valves which are difficult to see in fresh specimens but appear clearly in fixed and cleared ones. The intestine has a well developed cardia or dilatation at its anterior end but soon narrows to a width less than half that of the body. The course of the intestine is very characteristic. Up to the middle of the body it runs on the left side, tending to be dorsal in position. In the region of the vulva it suddenly turns towards the right side passing in between the blind ends of the ovaries and pressing the uterus below it, and, for the rest of its course runs on the right side of the body, tending to be more ventral in its position. This disposition of the intestine is a constant feature for all female worms. As a result of this the genital organs lie on the right side of the intestine in the anterior half of the body and on its left side in its posterior half.

Seen in a lateral view, the intestine appears to run above the genital organs in one half of the body and below them in the other half (Fig. 2, G, H). The rectum is very small and the muscles ordinarily attached to the rectum are very poorly developed. The anus is situated at a distance of 110$\mu$ from the posterior end of the body. The tail is pointed, tapering gradually to its end.

The nerve ring lies just anterior to the posterior oesophageal bulb and is 115$\mu$ from the anterior end of the body. The excretory pore is situated in the region of the posterior oesophageal bulb and is connected with a glandular cell which in most cases lies behind the base of the oesophagus.

The genital organs are paired. The anterior ovary arises as a club-shaped thick knoblike structure in the region of the vulva or slightly anterior to it. It runs anteriorly in a dorsal position for a considerable distance and becomes reflexed before reaching the base of the oesophagus, 70$\mu$ behind it in the worm under consideration, and opens near this place into a short oviduct which runs posteriorly and ventrally, finally opening into the uterus. The latter continues posteriorly and is connected with the uterus of the other side in the region of the vulva. The posterior ovary also arises as a club-shaped knob in the region of the vulva and runs posteriorly, being reflexed before reaching the level of the anus, continuing into the oviduct which runs in an anterior direction and finally opening into the uterus. The latter is continuous with the uterus coming from the anterior side. In younger females (Fig. 2, G) the uteri contain either unsegmented eggs or eggs which have already started segmentation but contain only a few cells, and the walls of the uteri can be made out. In fully developed worms the number of eggs contained at one time in the uteri increases, with the result that the uteri press the intestine and the ovary hard and fill nearly the whole of the body space (Fig. 2, H). In many such cases two rows of eggs can be seen in the uteri. At one time the uteri may contain as many as 30 fully formed eggs. In older females the eggs may be seen in all stages of development but generally they are laid before being embryonated, the worms being oviparous. However, in moribund females eggs may even hatch while in the uterus and numerous larvae may be seen wriggling actively within the body as is shown by Looss (7). The eggs have very thin shells and are 42 to 48$\mu$ long by 23 to 30$\mu$ wide when laid (Fig. 2, K). They are smaller than those laid by the parasitic female.

When all the eggs produced by the ovaries are passed out of the uterus, the genital tubes shrink in size but their outlines are still clearly visible. After reaching this stage the worms usually die within a week. Individual "post-functional female" (Fig. 2, J) worms have been kept alive in culture up to 12 days.

The vulva is situated in the middle of the body, at a distance of 515$\mu$ from the anterior end. It is slightly salient and is in the form of a transverse slit guarded by two distinct lips (Fig. 2, E, F). The bases of the lips are connected with the body with radial muscles which probably control the opening of the

vulva. The pattern of the muscles appears to be similar in all worms. The vagina is very weakly developed and runs transversely inwards into the uterus. It occupies a little more than half of the body width.

Table I gives an idea of the variations in size and ratio of the different parts of the worms.

TABLE I

MEASUREMENTS OF 10 FEMALES OF THE FREE-LIVING GENERATION

| No. | Length of oesophagus | Distance of vulva from anterior end | Distance of anus from post. end | Total length | Distance of anterior ovary from base of oesophagus | Distance of post. reflex of ovary from anus |
|---|---|---|---|---|---|---|
| 1 | 130$\mu$ | 375$\mu$ | 88$\mu$ | 770$\mu$ | 60$\mu$ | 70$\mu$ |
| 2 | 130 | 420 | 80 | 840 | 80 | 85 |
| 3 | 98 | 310 | 85 | 640 | 70 | 90 |
| 4 | 128 | 450 | 125 | 910 | 85 | 110 |
| 5 | 172 | 510 | 100 | 960 | 60 | 75 |
| 6 | 122 | 420 | 115 | 900 | 70 | 80 |
| 7 | 150 | 570 | 120 | 1110 | 90 | 90 |
| 8 | 130 | 420 | 145 | 970 | 70 | 120 |
| 9 | 140 | 515 | 110 | 1010 | 70 | 80 |
| 10 | 170 | 500 | 100 | 945 | 65 | 75 |

*Male* (Fig. 1, I)

On the average the males are smaller in length than the females and are comparatively more slender. The body is more or less subcylindrical and tapers both anteriorly and posteriorly. Anteriorly the tapering is gradual and starts from about the middle of the body. Posteriorly the body suddenly tapers behind the anus and forms a slender pointed tail. The length of the worms varies from 700 to 825$\mu$. However, in the following description measurements are taken from a worm 800$\mu$ in length and having a maximum width of 50$\mu$. Striations of the cuticle are so fine that they can only be seen under the oil immersion lens. The head is distinctly separated from the body by a constriction, and is 8$\mu$ high (Fig. 1, K); width of the body at this constriction is 12$\mu$. The head appears in the form of a crown and in an *en face* view (Fig. 1, J) shows structure similar to that of the female. The form of the buccal cavity is also the same as in the female.

The oesophagus is 130$\mu$ long and is constituted as follows: a small anterior muscular vestibule 12$\mu$ long by 12$\mu$ wide; a club-shaped anterior rhabditoid bulb with a length of 62$\mu$ behind the vestibule and a maximum width of 22$\mu$ near its base; a narrow isthmus, 29$\mu$ long and 8$\mu$ wide which merges imperceptibly into a posterior valvular bulb. The latter is 27$\mu$ long by 22$\mu$ wide. The nerve ring lies just anterior to the oesophageal bulb. The oesophagus opens into the intestine through a pair of valves. The intestine is dilated

anteriorly to form a slight cardia. The anus is situated at a distance of 65$\mu$ from the posterior end. The body behind the anus tapers suddenly, resulting in a small and slender pointed tail. The excretory pore is situated in the region of the oesophageal bulb and is internally connected with a glandular cell.

There is a single testis which ends blindly, about 20$\mu$ behind the base of the oesophagus. In this species it neither crosses the base of the oesophagus nor is it reflexed as is shown by Kreis (6) for *S. simiae* and by Faust (4) for *S. stercoralis*. It is a stout cylindrical structure and occupies nearly the whole width of the body, pushing the intestine to one side. It always lies on the right side of the body. The testis portion appears to run for about 155$\mu$ and near the middle of the body continues into the vas deferens. The latter is as broad as the testis itself but distinction between the two can easily be made by a careful observation of freshly killed worms. Posteriorly the vas deferens narrows to form an ejaculatory duct which opens into the cloaca. There are two spicules, very much like the spicules of rhabditids in shape. They are arcuate, each about 33$\mu$ in length. They appear as curved channeled gutters, forming a knoblike structure at the beginning and gradually narrowing down to a pointed end. The sides of the spicules are raised in the form of triangular alae just after the constriction of the knoblike end. Both the spicules are similar in structure and are equal in size. A gubernaculum is present. It is 20$\mu$ long and has a width of 2.5$\mu$. It is a flattened structure, folded upon itself and acting as a channel to guide the spicules and support them in their movement. In half-conscious living worms, movement of the spicules in this channel can be observed. The sides of the gubernaculum are quite broad but both proximally and distally they taper down to fingerlike blunt ends.

Most of the workers on *Strongyloides* describe only two pairs of caudal papillae, one pair preanal and one pair postanal. These two pairs are very clearly seen. Kreis (6) describes one preanal and two postanal pairs in *S. simiae*, but refers to his additional pair as doubtful. However, in the study of a ventral view of the tail (Fig. 1, L) of *S. papillosus*, four pairs of papillae could be seen. Two pairs are preanal; the first pair lies about 45$\mu$ anterior to the cloacal opening; and the second pair lie about 10$\mu$ anterior to the cloaca. The other two pairs are postanal; the first pair consisting of very small papillae which lies just behind the cloacal opening with the other pair lying about 27$\mu$ posterior to it. The anterior pair or postanal papillae as described by Kreis (6) for *S. simiae* could not be seen. It is possible that more papillae might be present on the tail which could not be observed because of their small size and the difficulty of preparing a satisfactory ventral view of the tail owing to the smallness of the worms and the strength of the curvature of the tail.

Table II gives an approximate idea of the variations in size of different parts of the body in different worms.

TABLE II

MEASUREMENTS OF 10 MALES OF THE FREE-LIVING GENERATION

| No. | Length of oesophagus | Distance of anus from post. end | Distance of anterior end of testis from base of oesophagus | Total length |
|---|---|---|---|---|
| 1 | 105$\mu$ | 65$\mu$ | 20$\mu$ | 825$\mu$ |
| 2 | 105 | 50 | 20 | 815 |
| 3 | 105 | 60 | 30 | 800 |
| 4 | 100 | 50 | 25 | 730 |
| 5 | 105 | 55 | 25 | 780 |
| 6 | 110 | 60 | 20 | 815 |
| 7 | 100 | 55 | 30 | 755 |
| 8 | 106 | 60 | 25 | 820 |
| 9 | 108 | 60 | 20 | 800 |
| 10 | 100 | 50 | 25 | 725 |

## Development of the Infective Larvae from the Eggs of the Parasitic Female

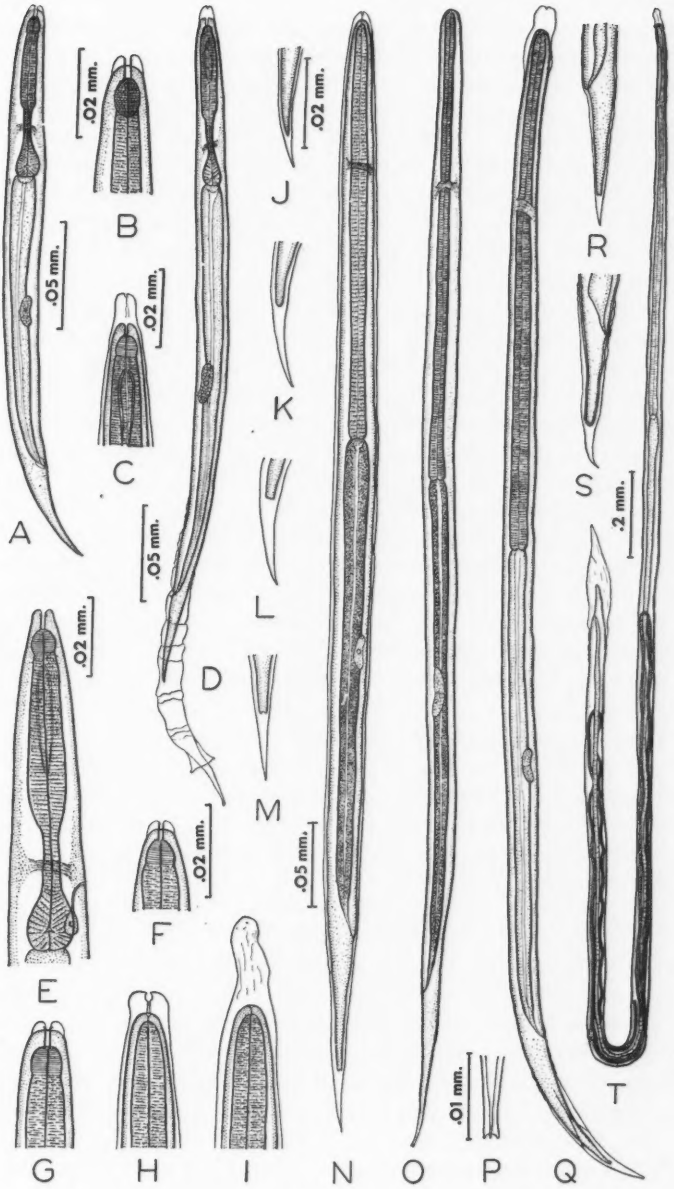
*First-stage Larva*

Newly hatched larvae (Fig. 3, AC) are all similar in morphology and no difference could be made between those which would develop into free-living adults and those which would go to form the infective larvae. But as the larvae grow and approach the first molt, differences can be made out between them. In those which are going to form infective larvae, the genital primordium remains unchanged; it neither increases in size, nor does its cell divide, while in the larvae going to form the free-living adults, the cell of the genital

FIG. 3. *The free-living and the parasitic phases of Strongyloides papillosus.*

*The direct cycle:*

*A.* *Rhabditiform larva, just hatched, lateral view.*
*B.* *Rhabditiform larva, just hatched, anterior view.*
*C.* *Rhabditiform larva, anterior end, at the time of the first molt.*
*D.* *Rhabditiform larva in process of the first molt.*
*E.* *Anterior region of the larva just after the first molt.*
*F.* *Beginning of separation of cuticle on head for the second molt.*
*G–I.* *Progressive increase in the separation of cuticle on head as the second molt approaches.*
*J–M.* *Progressive separation of cuticle on tail, also showing the gradual modification of the tail from a pointed to a notched end.*
*N.* *Larva during the process of the second molt.*
*O.* *Third stage or infective larva.*
*P.* *Tail end of infective larva.*
*Q.* *Infective larva in process of the third molt, in the lung of the host.*
*R–S.* *Progressive separation of cuticle on the tail of the fourth stage larva in the intestine of the host, also showing the change in the form of tail from a notched to a blunt finger-shaped end.*
*T.* *Larva undergoing the fourth and the last molt in the intestine of the host.*


A
.05 mm.
B
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C
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E
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F
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G
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J
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K
L
M
.05 mm.
N
O
.01 mm.
P
Q
R
S
.2 mm.
T

FIG. 3.

primordium begins to divide, with the result that at the time of the approach of the first molt it consists of a number of cells and has considerably increased in length. The first molt takes place, as does the first molt of the larvae of the direct generation, between the ages of 7 and 10 hr. after hatching (Fig. 3, D).

*Second-stage Larva*

After the first molt has taken place, the second-stage larvae can be easily distinguished from the first-stage by the absence of any separation of cuticle on the head region (Fig. 3, E). The larvae have a length of 325 to 400$\mu$ and there is no perceptible difference in their morphology as compared to their previous stage.

The larvae gradually grow in length and, as they grow, the oesophagus also elongates and progressively loses its rhabditoid character. A few hours after the first molt the cuticle on the head and tail region begins to separate again (Fig. 3, F). The tail appears to retract a little and becomes blunt instead of being pointed (Fig. 3, J, K). The blunt ending then flattens at the tip (Fig. 3, L) and gradually becomes forked (Fig. 3, M). This whole process takes place after the cuticle has already separated from the tail. It takes several hours and the different stages of this transformation can be easily observed. In contrast to the first molt, the second molt is not as quick but is more persistent and can be easily observed in most of the larvae. At the age of about 26 to 28 hr. larvae are seen in the process of molting (Fig. 3, N). In the majority of cases the major portion of the casting sheath is passed by the tail end and only a small caplike portion by the head; but larvae have been observed in which at least half of the sheath was passed through the head end only.

Sandground's observation that "the notch in the tail is formed by the rupture of the tail at the time that the filariform larva emerged from the old cuticle" is not confirmed for the present species. When the cuticle separates from the tail, the latter is still pointed and it is long after that stage that it gradually modifies its shape and structure. The larvae at the time of the molt measure about 600$\mu$ in length.

*Third-stage or Infective Filariform Larva*

The size of the infective larvae (Fig. 3) varies with the cultures in which they are grown. In normal sheep faecal cultures they tend to be a little longer than in artificial cultures. However, the body is always very slender and considerably longer in proportion to its width. The larvae are very active and move about vigorously. They are 575 to 640$\mu$ long and about 16$\mu$ in maximal width. The body tapers slightly but gradually, anterior to the nerve ring. The head is rounded and blunt and is about 7$\mu$ broad. Posteriorly the body tapers slightly but gradually behind the middle, ending in a long slender tail. The tail appears to be trifid at its end, each ray ending in a swollen knoblike structure. The structure of the tail is a little confusing; in most larvae it appeared to be trifid while in others it appeared to have four endings.

The head is not separated from the body as it is in the free-living adults. The buccal cavity of the rhabditiform larvae almost disappears. The oesophagus nearly reaches the anterior end and the mouth opening communicates with it through a minute funnel-shaped cavity. The oesophagus has become very elongated and occupies about 40% of the total body length. It has nearly lost its bulbs and rhabditoid contours. However, there is a small local swelling of the oesophagus a little anterior to the nerve ring. Behind the nerve ring the oesophagus gradually increases in width and is broadest near its base. No true posterior bulb is present. The broad base of the oesophagus fills nearly about three-fourths of the body space and may represent the remnants of the posterior bulb of the previous larval stage. The intestine is slender and has a width of about 8$\mu$ near its beginning, but in its posterior half it gradually becomes thinner so that where it joins the rectum it is only 4$\mu$ wide. The rectum is in the form of a very fine capillary tube. The anus lies at a distance of 78$\mu$ to 94$\mu$ from the posterior end of the body. The tail is long and slender and occupies a little less than one-sixth of the total body length.

The nerve ring lies slightly behind the anterior third of the oesophagus, 78 to 92$\mu$ from the anterior end of body. The excretory pore is difficult to see; it opens a little behind the nerve ring. The genital primordium now consists of two cells and is about 25$\mu$ long. It lies slightly behind the middle of the body, 323 to 362$\mu$ from the anterior extremity.

## Development of the Infective Larvae from the Eggs of the Free-living Unisexual Adults

At the time of deposition, the eggs are in their early stages of segmentation. They hatch in about 6 to 10 hr. and the newly hatched larvae are exactly the same as those which hatch from the eggs of the parasitic female. Their transformation to the infective larvae is also similar and is accompanied by two molts. The only difference between the development of these larvae and those from the eggs of the parasitic female is that in the latter some of the larvae grow to form infective larvae while others grow into a generation of free-living sexual adults; in those from the free-living female all the larvae are transformed into infective larvae.

## Development of the Infective Larvae in the Host

To study the growth of the larvae inside the host, four rabbits were infected percutaneously and postmortem examinations were made at different intervals to study the behavior and development of the larvae.

Rabbit No. 1 was killed and examined 32 hr. after the application of the larvae to the skin. Larvae were found in the heart, in the lungs, and in the trachea. Two larvae in the lungs were found in the act of casting their cuticle (Fig. 3, Q). These larvae are slightly larger than third-stage infective larvae and the tail inside the casting skin is still bifurcate; otherwise in general morphology they are similar to the infective larvae.

Rabbit No. 2 was killed 75 hr. after infection. No larvae were found in the heart. A few larvae were recovered from the lungs and a few from the trachea and oesophagus. Numerous larvae were found in the small intestine, coiled inside the mucosa.

Rabbit No. 3 was killed and examined five days (120 hr.) after infection. A few larvae were found in the lungs, in the trachea, and in the oesophagus, and numerous larvae in the small intestine in all stages of development.

Rabbit No. 4 was killed seven days after infection. No larvae or adults were recovered from the lungs or from the trachea. The small intestine showed a heavy population of worms in all stages of growth from the infective larvae to the fully grown adults with eggs already developed in the uterus.

*Fourth-stage Larva*

Fourth-stage larvae are found in the trachea, the oesophagus, and the intestine after about 36 hr. of initial infection. Early larvae of this stage are similar in morphology to the infective larvae, except that they have grown slightly in size and the nerve ring has become more pronounced. They are quite active when placed in physiological saline but this activity does not last long and they exhibit a tendency to become sluggish and coil and uncoil themselves in the fashion of a watchspring as reported by Faust (4) for the "post filariform larvae" of *S. stercoralis*, which he recovered from the lungs. They measure 680$\mu$ to 800$\mu$ in length. The oesophagus has the same ratio to body length as in the case of infective larvae. It measures 310$\mu$ to 360$\mu$ in length. Larvae recovered from the intestine 75 hr. after infection were in the same stage as those in the lungs and had nearly the same measurements. Those recovered on the sixth day (120 hr.) of infection were in all stages of development. Some were still in the early stages and looked like infective larvae, indicating that they had reached their destination very recently while others have been there for some time. Growth of the larvae in the intestine starts about 75 hr. after initial infection of the animal. After this the larvae grow rather rapidly and separation of the cuticle on the tail starts indicating the approach of the next molt. On the sixth day larvae in all stages of molting can be seen. Even after the separation of the cuticle at the tail end has started, the latter remains forked for some time (Fig. 3, R); but the end gradually becomes thicker and the forked structure becomes "fingertip-shaped". (Fig. 3, S). In the meantime the worm has elongated progressively and has attained a size of about 3.5 mm. The genital primordium divides and grows very actively and within this short period forms a complete outline of the genital organs as arranged in the adult, but the ovaries are still filamentous and the uterus has not yet developed a lumen. Near the middle of the body, a padlike convex thickening of the body wall is developed in which a slit appears to arise from outwards. This is the future vulva. This whole growth presses into the uterus and finally opens into it.

Larvae were seen casting their molt at the age of 120 hr. after infection (Fig. 3, T). They were between 3.0 to 3.8 mm. in length and had a maximum

width of about 50$\mu$ in the region of the vulva. A larva 3.53 mm. long gave the following measurements: oesophagus 915$\mu$ long; tail 70$\mu$ from posterior end; nerve ring 180$\mu$ and vulva 2.4 mm. from the anterior end of body; loop of anterior ovary 460$\mu$ behind the base of oesophagus and that of the posterior ovary 220$\mu$ in front of the anus.

*Parasitic Adults*

Newly molted larvae look like young adults in which, although the reproductive organs are completely laid down, the eggs have not yet made their appearance. These soon attain full adulthood and start laying eggs. Eggs have been recovered from the faeces of an infected animal as early as the seventh day after infection. Adults were only found in the small intestine, mainly in its upper part. No adult worms were recovered either from the lungs or from the trachea. Even if the larvae were delayed as long as six days during their migration and were discovered in the lungs and the trachea, they were still like the infective larvae in shape and size, although others, given at the same time but which had completed the migration earlier, had nearly reached maturity in the intestine. Thus it appears that, in *S. papillosus*, worms grow to adulthood only in the intestine and do neither grow in size nor change their morphology till they reach this destination.

The body of an adult parasitic female (Fig. 4, A) is long and slender. In *S. papillosus* it is comparatively longer than in other species of this genus. In the 25 worms measured, it varied between 4.78 to 5.85 mm. in length. It is more or less cylindrical in shape. It tapers slightly in the oesophageal region and ends in a rounded head which is about 15$\mu$ wide. The posterior end also tapers gradually from behind the flexure of the posterior ovary and ends in a fingertip-like blunt tail. The greatest width of the body is in the region of the vulva and varies between 50 and 65$\mu$. In the following description measurements are taken from a worm which had a length of 5.41 mm. and a maximum width of 60$\mu$.

The cuticle is thin and does not show any obvious striation or annulation, but faint cross lines may be seen under very high magnification. The anterior end of the body is round and truncated and is not separated to form a distinct head structure like that of the free-living generation. An *en face* view (Fig. 4, B) shows that the mouth is surrounded by four small lips, each of which is separated from the other by a deep cuticularized inlet, so that the mouth opening appears to be four-partite. There are four cephalic papillae, two dorsolateral and two ventrolateral in position. Amphids appear as comparatively large openings and are situated laterally. There is no distinct buccal cavity. The mouth opening appears to communicate directly with the oesophageal lumen. The oesophagus occupies about one-seventh of the total body length. It is slightly claviform and has its maximum width of 40$\mu$ at its base; near its beginning it is only about 16$\mu$ wide. The intestine does not show any marked dilatation at its beginning. It runs straight for a short distance of about 120$\mu$ and then is caught in the coils of the loops of the anterior

ovary. Behind these coils it again runs straight but lies on the left side of the body, the right side being occupied by the ovary. A little anterior to the region of the vulva it bends and passes over to the right side in between the blind ends of the ovaries, and runs straight backwards, this time lying on the right side of the body till it is taken up into the coils of the posterior ovary. The latter runs on the left side of the body. Behind the flexure of the posterior ovary it runs straight and opens into a short rectum. The anus is situated at a distance of 32$\mu$ from the posterior end of the body. The tail is short and ends in a "finger-tip-like" blunt point. This shape is typical of *S. papillosus* and allied species and is an important taxonomic feature.

There are two ovaries, one anterior and the other posterior. Both are reflexed and arise a little anterior to the vulva. The blind end of the anterior ovary is directed posteriorly and is knoblike. This ovary runs anteriorly on the right dorsal side of the body in a straight course till it is reflexed at a point 120$\mu$ behind the oesophagus. Then it runs backwards for a considerable length, ultimately opening into a short oviduct which communicates with the uterus. The uterus is short in comparison with the length of the ovary, and contains on an average about 10 eggs arranged longitudinally in a single row. For a length of about 550$\mu$ behind the anterior flexure, the two limbs of the anterior ovary are coiled round the intestine. Usually there are two and a half such coils but the number of the coils varies between one and one-half to three and one-half, probably depending on the age of the worm. The posterior ovary arises in the region of the blind end of the anterior ovary and runs posteriorly, becoming reflexed at a distance of about 120$\mu$ anterior to the anus, then runs anteriorly, opening into the oviduct which in turn communicates with the uterus. The limbs of the posterior ovary also coil round the intestine for a distance of about 500$\mu$ anterior to the flexure. They too form one and one-half to three and one-half coils. The anterior and the posterior uteri meet in the region of the vulva. The vulva communicates with the uterine cavity through a short transverse vagina, which occupies more than half of the body width. There is no ovejector.

The vulva lies somewhere between the middle and the posterior third of the body, 3.09 mm. from the anterior end in the worm under study. It is in the form of a transverse slit and is guarded by two distinct lips which are supplied with radial muscles (Fig. 4, C, D). In the living worm these lips are not salient but after fixation they appear to be slightly raised.

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FIG. 4. *The parasitic phase of Strongyloides papillosus.*

*The parasitic female:*

*A. Adult, lateral view.*
*B. En face view.*
*C. Vulvar region, lateral view.*
*D. Vulvar region, ventral view.*
*E. Tail, lateral view.*
*F. Egg as passed out with the faeces.*

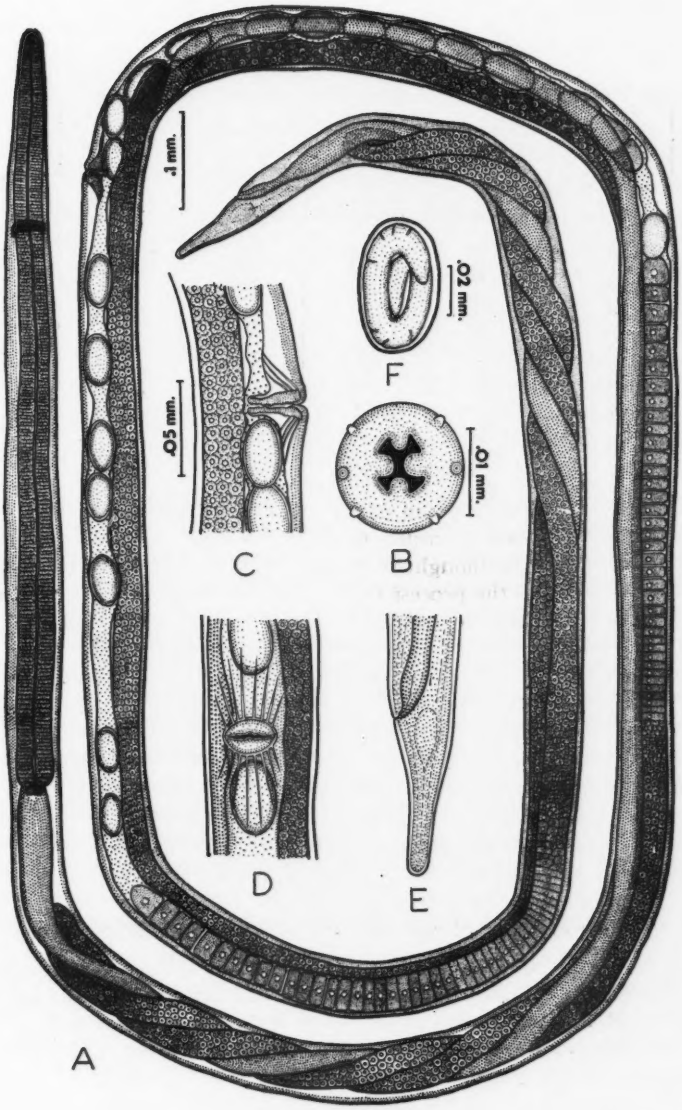


FIG. 4.

Table III gives an idea of the variations in size and proportion of the different parts of the body in different worms.

TABLE III

MEASUREMENTS OF 10 PARASITIC FEMALES

| No. | Length of oesophagus | Distance of anus to posterior end | Base of oesophagus to anterior flexure of ovary | Posterior flexure of ovary to tip of tail | Vulva to anterior end | Total length |
|---|---|---|---|---|---|---|
| 1 | 820$\mu$ | 67$\mu$ | 70$\mu$ | 220$\mu$ | 3150 mm. | 5230 mm. |
| 2 | 950 | 78 | 160 | 160 | 3500 | 5550 |
| 3 | 870 | 63 | 100 | 210 | 3330 | 5620 |
| 4 | 950 | 65 | 120 | 210 | 3500 | 5850 |
| 5 | 920 | 68 | 150 | 250 | 3540 | 5720 |
| 6 | 820 | 54 | 160 | 200 | 3430 | 5150 |
| 7 | 780 | 72 | 160 | 180 | 3020 | 5000 |
| 8 | 720 | 59 | 90 | 150 | 2860 | 4780 |
| 9 | 730 | 61 | 100 | 210 | 2860 | 4850 |
| 10 | 830 | 58 | 90 | 210 | 3140 | 5370 |

No parasitic males were found during the present study. Females of the genus *Strongyloides* were regarded as parthenogenetic till Sandground (11), after observing what he thought to be sperms inside the body of females and what he thought to be the process of fertilization, suggested that these worms may be syngonic. Kreis (6) and Faust (4) reported the occurrence of parasitic males but the males they described had the morphology of the males of the free-living generation. It is doubtful whether these are the normal males of the parasitic generation, as those which have been reported in the allied genus *Parastrongyloides* possess an oesophagus like that of the female. Graham's (5) experiments with single larva infections proved that males were not necessary for the production of viable eggs by the female but the question remained whether the females were syngonic or parthenogenetic. Chitwood and Graham (3), as a result of the examination of the eggs of *S. ratti*, reported that the eggs of the parasitic female are parthenogenetic.

## Discussion of the Life History of *S. papillosus*

Although life history studies of members of the genus *Strongyloides* have engaged the attention of numerous workers, very few observations have been made on the molting of the larvae. The heterogonous development in the free-cycle and the migration of the larvae inside the body of the host make this study more complicated.

Most workers have mentioned only one molt during the course of development of the infective larvae of various species of *Strongyloides*. Looss (7), Lucker (8), and Matoff (9) observed two molts during the development of the infective larvae of *S. stercoralis*, *S. ransomi*, and *S. papillosus* respectively;

the observations of the present writer confirm their findings. That the first molt has been apparently missed by most of the workers is understandable as it is difficult to observe owing to the thinness of the cast sheath and the suddenness with which it is cast.

In the development of the free-living sexual adults from the eggs of the parasitic females, all workers except Lucker (8) and Matoff (9) report only a single molt. Lucker observed the usual four molts while Matoff could observe only two. My findings agree with those of Lucker.

During the parasitic phase of the life cycle no worker until now has reported more than one, the final, molt which takes place in the intestine of the host when the worm assumes the morphology of the adult female. However, Lucker (8), working with *S. ransomi* of the pig, writes "These facts are a strong indication that the entire development from the smallest larva found in the intestine to the adult form occurs in connection with but one molt. It cannot, however, be concluded with certainty that only one molt occurs during the parasitic development of *S. ransomi*, since it is possible that following percutaneous entry to the host the larvae may molt while they are in the lungs. However, none of the larvae recovered from the lungs of experimental animals were molting."

As a general rule the number of molts that occur during the development of a nematode is four. In those in which the development is direct all four molts occur in the body of the host while in those where there is a free-living infective stage the larvae molt twice outside the host and twice inside its body. Even in those with an intermediate host, two molts occur in the vector and two in the definitive host. Considering these facts and also the number of molts undergone by the free-living sexual generation of *Strongyloides*, it seems probable that two molts should occur in the body of the host.

Looss (7) attempted to explain the occurrence of four molts in *Strongyloides* in a very interesting way. He writes that "in numerous experiments made with three different species (including *S. stercoralis*), I have observed that the descendants of the parasitic form, if they develop into the sexually differentiated stage, undergo one molt during their free-life, while their descendants, during their life in the ground, undergo two molts, the first connected with but hardly noticeable changes in shape and organization, the second connected with the metamorphosis into 'filariform' larvae. These latter, after having become parasites, cast their skin a third time and thereby assume the shape of the full grown parasites. We here thus have again four molts between every two parasitic stages." Thus Looss saw only four molts where he should have seen eight.

However, the present study shows clearly that molting in the genus *Strongyloides* follows the general rule. There are four molts during the development of the free-living sexual adults, two molts during the development of the infective larva, and two molts in the development of the parasitic adult from the infective larva after its entry into the body of the host. One molt i.e. the third

molt, takes place in the lung and the other, the fourth, in the intestine. The larva just after the third molt is morphologically not very different from the infective larva and it still possesses a notched tail.

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# THE TICKS OF ALBERTA WITH SPECIAL REFERENCE TO DISTRIBUTION[1]

By John H. Brown[2] and Glen M. Kohls[3]


## Abstract

The material in this paper is the result of 11 years of collecting over the accessible portions of Alberta, with the exception of the settled area from Lac La Biche to Fort McMurray. The area north from Grimshaw to Fort Vermilion has not been surveyed in detail, and further collections will be made. Nine species are recorded: *Dermacentor andersoni* Stiles, *D. albipictus* (Packard), *Haemaphysalis chordeilis* (Packard), *H. leporis-palustris* (Packard), *Ixodes angustus* Neumann, *I. kingi* Bishopp, *I. sculptus* Neumann, *I. spinipalpis* Hadwen and Nuttall, and *Otobius lagophilus* Cooley and Kohls. Maps showing the known distribution of the several species in Alberta are presented.


## Introduction

In 1944, Brown (4) reported on the importance and the then known distribution of the spotted fever tick, *Dermacentor andersoni* Stiles, in Alberta, and also mentioned that nine other species had been recorded as occurring in the Province. Since that time intensive collecting under the supervision of the senior author has elicited much new information in regard to tick species and their distribution in Alberta. Some of this information was published by Bow and Brown (2) in 1945, and Brown and Roy (6) in 1943. Many of the collections on which the present and the earlier reports were based were identified by the junior author.

## Species Present

*Dermacentor andersoni* Stiles
*Dermacentor albipictus* (Packard)
*Haemaphysalis chordeilis* (Packard)
*Haemaphysalis leporis-palustris* (Packard)
*Ixodes angustus* Neumann
*Ixodes kingi* Bishopp
*Ixodes sculptus* Neumann
*Ixodes spinipalpis* Hadwen and Nuttall
*Otobius lagophilus* Cooley and Kohls

All of the above listed species, with the exception of *O. lagophilus*, have been collected in Alberta by the senior author and specimens are in the

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*Contribution from the Provincial Department of Public Health, Edmonton, Alta., and the Rocky Mountain Laboratory (Hamilton, Mont.), National Institutes of Health.*
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Division of Entomology, Alberta Department of Public Health collection. The presence of *lagophilus* has been recorded by Cooley and Kohls (8, 9) on the basis of specimens studied by them as discussed later in this report.

## Species Distribution

The accompanying maps depict the known distribution of the recorded species in Alberta.

*Dermacentor andersoni* Stiles

This tick, commonly known as the spotted fever tick, was reported from Alberta in 1915 by Hewitt (15). Hearle (14) in 1938 reported it as being present in southern Alberta. Gibbons (11) in 1939 also reported this tick. In 1943 Bow and Brown (1) and Brown (3) showed its relationship to tularemia in southern Alberta, and in 1944 Brown (4) reported on its importance and distribution in the Province. Since 1938 over 100,000 host and drag ticks have been collected and the records show that the distribution (Map 1) is substantially the same as reported by Brown (4) in 1944, but with an extension north in the Rocky Mountain region to Jasper through an area not well covered in earlier surveys.

*Dermacentor albipictus* (Packard)

The winter tick is generally believed to be confined to the mountainous and northern parts of the Province where it is said to infest moose and other ungulates such as deer and elk. However, only three collections have been seen by us. These were from Edson-Jasper, Jasper, and Banff, the hosts being moose, horses, and moose, respectively. Strickland (16) reports an infestation occurring on moose at Elk Island Park in 1939. Map 2 shows the localities where the species has been found in the Province. The tick is probably much more widely distributed than our records indicate.

*Haemaphysalis chordeilis* (Packard)

The bird tick was reported by Hearle (14) in 1938 under the name of *H. cinnabarina* Koch as being "recorded mainly from Manitoba, although it is known also from Alberta and British Columbia," but he does not cite any Alberta records. The senior author collected two males and five females as "drag" ticks near Medicine Hat on May 4, 1942 (Map 2). Subsequent collecting throughout the Province has failed to yield further specimens.

*Haemaphysalis leporis-palustris* (Packard)

The distribution of the rabbit tick in Alberta does not appear to coincide with that of its usual hosts, members of the family Leporidae, which occur throughout most of the Province (Map 3). Nor is it always the tick most commonly found on rabbits within its range, since in Alberta south of Calgary, *D. andersoni* was the species most frequently taken on these hosts.

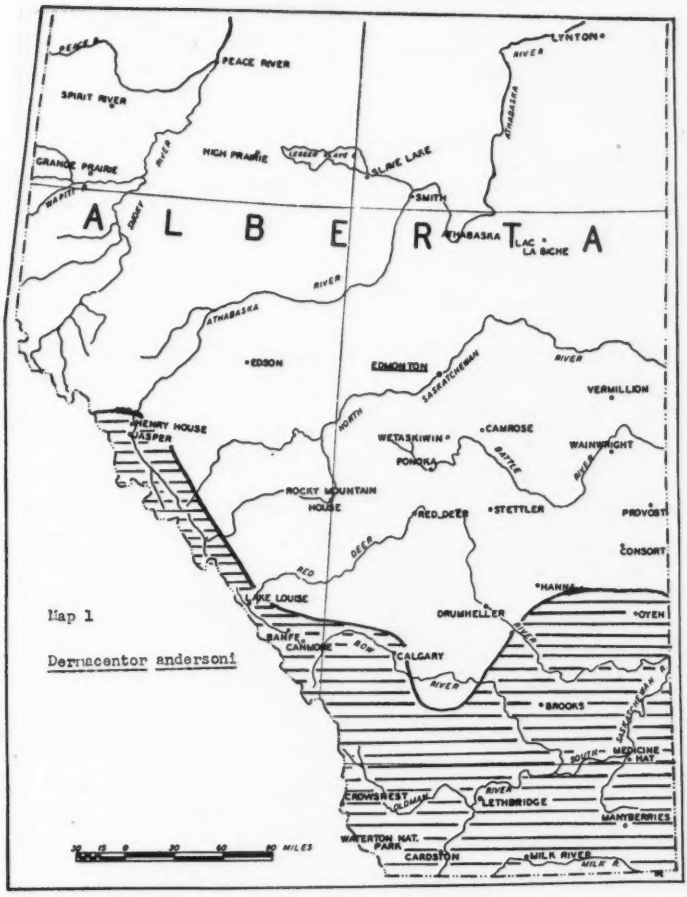


FIG. 1.

Over 100 immature and adult specimens have been recovered during the past 10 years. These have been taken from jack rabbits (*Lepus townsendii*), bush rabbits (*Lepus americanus*), grouse (*Canachites canadensis*), bush partridge (*Bonasa umbellus*), ravens (*Corvus corax*), magpies (*Pica pica*), and Richardson's ground squirrel (*C. richardsonii*). Brown (5) in 1945 reported the recovery of an engorging adult from the arm of a man at Camrose and Strickland (16) recorded its having been found on a turkey at Lavoy in 1927.

*Ixodes angustus* Neumann

One adult female was collected from a red-backed mouse, *Clethrionomys* sp., at Faust (Map 2) on the southwest shore of Lesser Slave Lake on Aug. 20, 1948. This species has not been previously reported for Alberta.

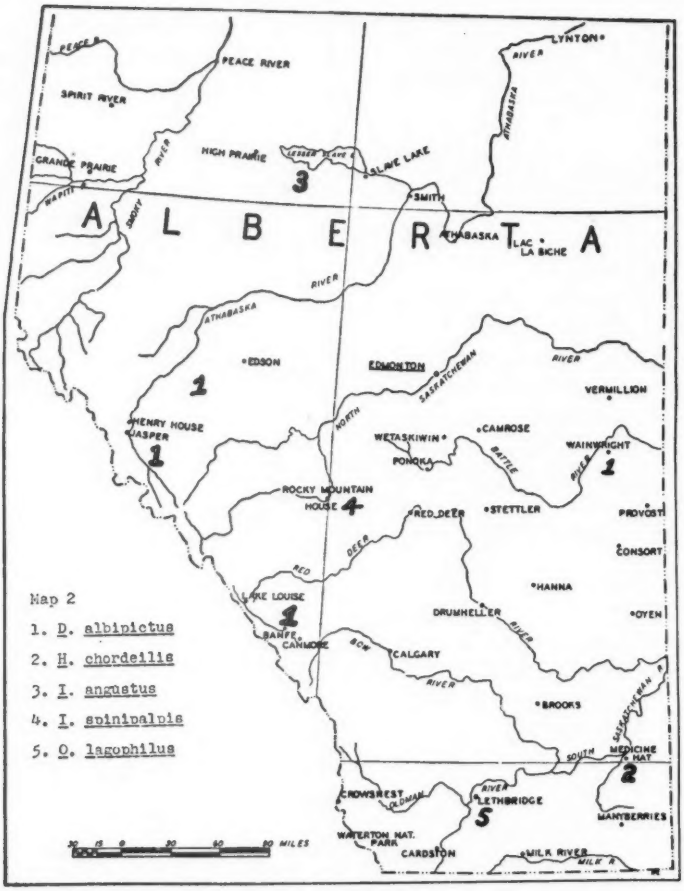


FIG. 2.

*Ixodes kingi* Bishopp

Under the synonymic name *I. pratti* Banks, Hadwen (12) reported two females of this species from dog and cat at Milk River, July 1911. Hearle (13) in 1938 recorded it from burrows of Richardson's ground squirrel at Medicine Hat, and Cooley and Kohls (10) recorded two males and two females from a badger (*Taxidea taxus*), at Suffield, September 1944. A total of 161 immature and adult specimens have been collected by the senior author since 1940. The distribution is shown on Map 4.

The known hosts in Alberta are badger and Richardson's ground squirrel.

*Ixodes sculptus* Neumann

This tick was first recorded from Alberta by Brown (4) from Richardson's ground squirrel at Medicine Hat in 1942. Since then over 2500 immature

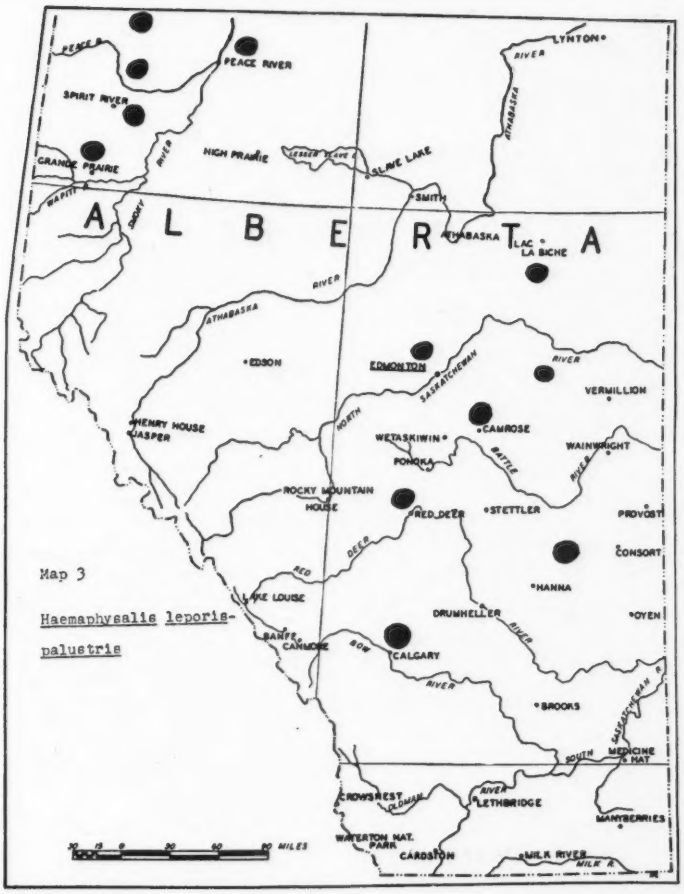


FIG. 3.

and adult specimens have been collected. The distribution is recorded on Map 5. The known hosts for this tick in Alberta are weasel (*Mustela frenata*), Richardson's ground squirrel, and Columbian ground squirrel.

*Ixodes spinipalpis* Hadwen and Nuttall

One specimen was collected from a shrew, probably *Sorex cinereus*, at Rocky Mountain House (Map 2) on Aug. 7, 1947. The species has not been previously reported for Alberta.

*Otobius lagophilus* Cooley and Kohls

There are four known Alberta collections of this tick, all from Lethbridge (Map 2). The species was first reported for the Province by Cooley and

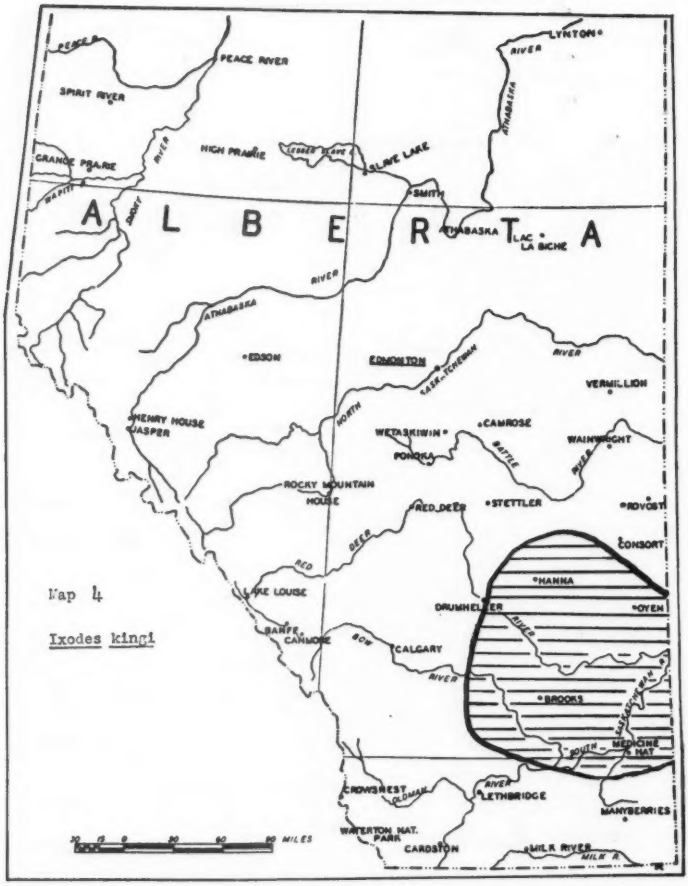


FIG. 4.

Kohls (8) who state: "Dr. S. Hadwen (1913) recorded a tick from jack rabbits from Lethbridge, Alberta, Canada, which had been identified as *Ornithodoros megnini*. Dr. Arthur Gibson, Dominion Entomologist, has recently sent us the single specimen referred to above, collected in 1912, as well as three others from a jack rabbit without date of collection." Two subsequent records reported by Cooley and Kohls (9) are as follows: one nymph from jack rabbit, Dec. 21, 1931 (E. H. Strickland), and one nymph from cat, Dec. 11, 1941 (H. L. Seamans). Despite intensive collecting in the Lethbridge area no further specimens have been found.

## Discussion

The distribution of *D. andersoni* is based on 11 years of collecting and its range on the prairie is exceptionally well determined. However, its

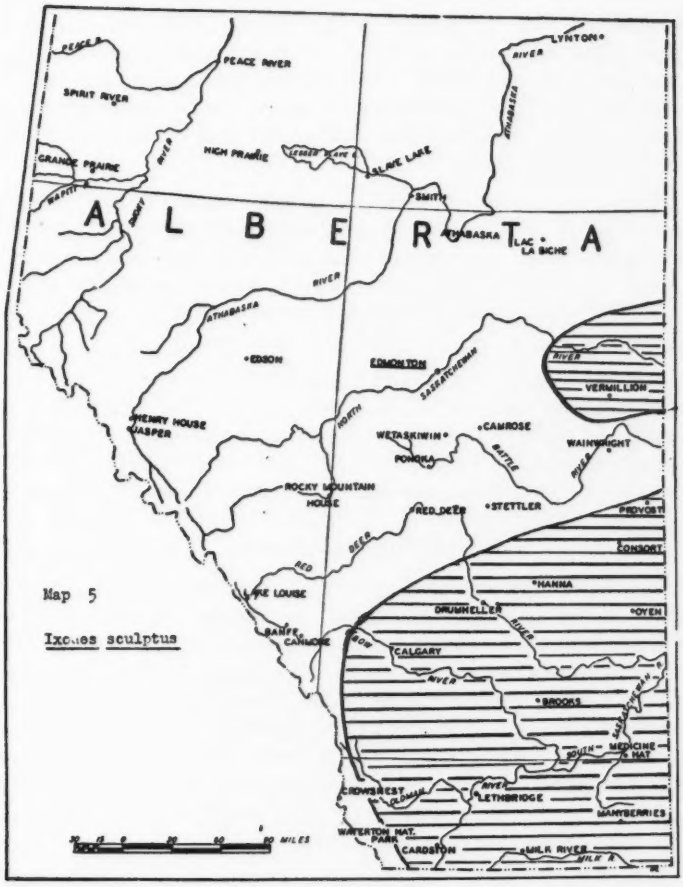


FIG. 5.

distribution in the foothills and mountains north of Banff has not been as well established, although specimens have been taken along the Banff–Jasper Highway and at Jasper. The area of greatest abundance for this species is in the southern part of the Province, being bounded on the north by the Medicine Hat – Crowsnest branch of the Canadian Pacific Railway. In the area north of the Red Deer River (Map 1) very few adults were found although larvae and nymphs were common on Richardson's ground squirrels.

The distribution of *H. leporis-palustris* (Map 3) did not fit the expected pattern, as it was thought that it was well-established throughout the Province. It should be stated, however, that during the collecting no special effort was made to establish its presence or absence on the Leporidae throughout the Province.

It is apparent that *I. sculptus* is the primary tick ectoparasite of the Richardson's ground squirrel, particularly in the eastern and south central portions of the Province (Map 5). Despite intensive collecting in the extreme southern part of the Province, this species does not appear to be at all common in that area. Here ground squirrels are infested with the larval and nymphal stages of *D. andersoni* chiefly.

The paucity of records of *D. albipictus* is rather surprising as it was thought that this tick was widely distributed in the mountainous and the northern parts of the Province. However, no special search was made for this species on livestock, particularly during the optimum winter season, and the lack of records may also be attributable in part to the fact that very few big game hunters report the presence of ticks on the game shot.

It is believed that further collecting, particularly in the Cypress Hills, and Rocky Mountains, and the wooded area northeast of Edmonton to Fort McMurray, will show that other species are present. Many mountain forms have been reported from British Columbia and it seems reasonable to assume that many of these are also present on the eastern slopes of the Rockies.

## Acknowledgments

Thanks are extended to Dr. R. A. Cooley, who identified some of the early collections before his retirement from the Rocky Mountain Laboratory, and to J. D. Gregson, Livestock Insect Laboratory, Kamloops, B.C. who also determined a number of specimens. Grateful appreciation is expressed for the assistance given by all members of the Alberta Survey crews in collecting and recording specimens. Without their assistance the work could not have been accomplished. The original drawing for the map of Alberta was prepared by W. H. Lawrence, Department of Forestry and Conservation, University of Michigan, Ann Arbor, while attached to the Division of Entomology for post-graduate work during the summer of 1947.

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